ANALYSIS OF HEMATOPOIESIS IN HUMAN NORMAL LONG-TERM MARROW CULTURES AND IN CULTURES FROM PATIENTS WITH CML AND AML

by

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ABSTRACT

The hematopoietic system supplies short-lived functional end cells of multiple lineages from a common pool of pluripotent stem cells throughout life. In man neoplastic transformation of these stem cells results in the development of the acute and chronic myeloid leukemias. An in vitro system where functional murine stem cells can be maintained for several months has recently become available. This system has been a powerful tool to analyze mechanisms regulating normal hematopoiesis and leukemogenesis in mice. The purpose of this work was to develop an analogous culture system applicable to human marrow and to evaluate its ability to support leukemic hematopoiesis.

Long-term cultures were established with normal human marrow cells. In these, the more primitive progenitors were found to be almost exclusively located in the adherent layer for the duration of the culture (i.e., at least 2 months). A prerequisite for these studies was the development of a procedure for detaching adherent cells so that various colony-forming progenitors could be assayed in semi-solid media. The consistent finding of adherent layer-associated hematopoiesis suggests that cell-cell interactions between primitive hematopoietic cells and adherent layer elements may be essential for the maintenance of the former.

Long-term cultures were also initiated with marrow from 11 CML patients and 13 AML patients (all untreated) and maintenance of normal and neoplastic progenitor cell populations assessed. A common finding was the rapid disappearance of neoplastic progenitor cells (recognized either by the presence of chromosomal abnormalities or by their abnormal differentiation capacity) in most cultures, even though cytogenetically normal precursors were often maintained. These differences between the behaviour of normal and

neoplastic cells in long-term cultures may reflect changes at the stem cell level that are related to the acquisition of abnormal growth properties. In a minority of patients a different result was obtained. Clonal dominance persisted in vitro and normal hematopoiesis was not detected. Thus long-term cultures have also allowed differences in the behaviour of primitive neoplastic cells from different patients to be revealed. Future investigation of the basis for these differences may provide new insights into the biological heterogeneity that characterizes these disorders.

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C H A P T E R I

THE HEMATOPOIETIC SYSTEM

1) GENERAL CONCEPTS

The hematopoietic system has been widely used as a model to study the regulation of cell proliferation and differentiation. This is due in part to the ease of repeatedly obtaining samples of blood and marrow cells from human or other animals, and to the fact that these cells can then be readily dispersed into single cell suspensions that contain cells at each maturation step of the hemopoietic differentiation pyramid, from the earliest stem cell to the most terminally differentiated blood elements. Similarly, cells whose impaired proliferation and differentiation give rise to a clinical picture of leukemia are also readily accessible using the same sampling procedures. On the other hand, the lack of structural organization associated with hematopoietic cell differentiation that allows all cell types to be obtained together in a single sample also poses a major problem for studies of these processes because pure populations of cells at a given stage of development are relatively difficult to isolate.

The production of mature blood cells takes place throughout the life span of the individual. The continuous turnover of relatively short lived mature cells is maintained by a class of more primitive "stem" cells with two major properties: that of unrestricted differentiation potential, i.e. the capacity to produce progeny of all of the blood cell lineages; and that of extensive self-renewal capacity, i.e. the capacity to produce new pluripotent cells (1). The multiple steps which occur in the bone marrow and lead to the formation of terminally differentiated cells from such pluripotent stem cells follow an

orderly sequence. As cells progress along a given pathway they acquire various unique features and at the same time their proliferative capacity decreases. This results in a hierarchy of different types of lineage-restricted precursors that can be identified and distinguished by the number and type of mature progeny they can each generate (Figure 1) (2,3). The most primitive cell that repopulates both the myeloid and lymphoid system in animals has a very high proliferative capacity. Although it has been possible to demonstrate the existence of such a cell in both mouse and human hemopoietic tissue there is as yet no quantitative assay that will allow their enumeration in a given sample.

Early experiments showed that adult mouse marrow contained stem cells able to repopulate irradiated mice with functional myeloid and lymphoid cells (4). Subsequently, it was shown that both myeloid and lymphoid organs could be repopulated by the progeny of a single bone marrow stem cell bearing a unique radiation-induced chromosome marker (5-7). Extension of these experiments by functional assays of the T and B lymphocytes found in the spleen of recipient animals transplanted with chromosomally marked stem cells provided evidence for a model in which pluripotent stem cells with myeloid and lymphoid differentiation potential exist. In addition, evidence of other derivative stem cell populations restricted either to the myeloid and B lymphoid lineages, or to the T lymphoid lineages was obtained (8,9).

In man, evidence for the origin of lymphoid cells from a pluripotent myeloid stem cell comes from analyses of the cells present in clonal neoplastic disorders (10): a single G6PD isoenzyme was demonstrated in circulating myeloid and lymphoid (B and T) cells of a patient with sideroblastic anemia (11) and the Ph^1-chromosome has been found in B cells and myeloid cells of most patients with chronic myelogenous leukemia (CML)(12,13). Recently, lymphoid blast crisis has been reported in a patient with CML whose blasts carried typical T

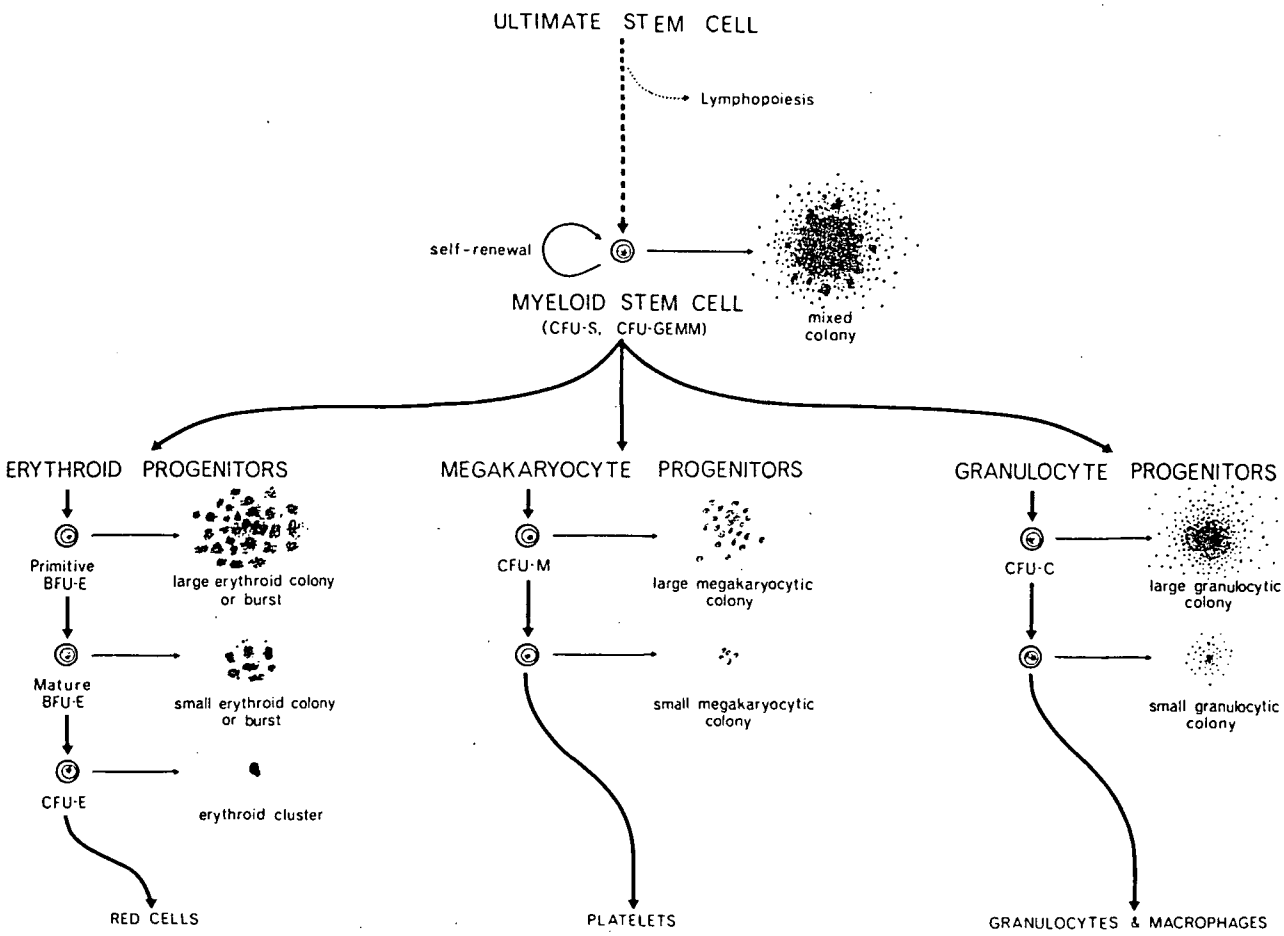

FIGURE 1. Schematic representation of the hematopoietic system as defined by colony-assays of pluripotent and committed progenitors (from Ref. 261).

lymphocyte markers, suggesting origin of the disease in this patient in a cell with T lymphocyte as well as myeloid differentiation potential (14).

Little is known about the mechanisms that regulate the proliferative capacity of pluripotent stem cells. During the life span of the normal individual this property does not appear to decline (15), although a progressive decrease in stem cell repopulating potential is seen when fetal liver, marrow and spleen cells in the mouse are compared (16). In the adult, decline in stem cell repopulating potential occurs following a variety of perturbations including serial transplantations (17,18), treatment with certain alkylating agents (19), sublethal irradiation (20), and the expression of certain W-locus allotypes (21). On the other hand, such decline may be prevented or reversed by infection with certain retroviruses (22), suggesting that regulation of proliferative capacity is not necessarily a fixed function of the mitotic history of the stem cell as has been proposed (23).

2) ASSAYS OF PROGENITOR CELLS

Bone marrow represents a mixture of hematopoietic cells belonging to various lineages at different stages of differentiation. The vast majority of hematopoietic cells are recognizable differentiated elements by well established morphological criteria, and less than 3% are more primitive progenitor cells (2). Besides the classically recognized hematopoietic compartments, the marrow also contains a variety of other cells. Some of these make up the "stroma" and are believed to play a role in regulating the hematopoiesis that, in the adult, normally occurs exclusively in the marrow. Progenitor cells are not morphologically recognizable by routine stains but can be identified and characterized using in vitro colony-assay methodologies. Such studies have shown that progenitor cells are found in the non-T non-B lymphocyte fraction,

where they co-purify with cells that in general look like medium-sized lymphocytes (24). Production and analysis of monoclonal antibodies that bind to progenitor cells have as yet failed to reveal any antigens that are unique to a given progenitor cell type. However, monoclonal antibodies specific for erythroid or granulocytic progenitors (25,26), and for subsets of CFU-S (27) have been recently generated. Physical methods of cell purification have also not been useful for the preparative isolation of any of the various types of progenitor cells, although as a group, they can be separated from more differentiated cells relatively easily (28). Thus, the allocation of progenitor cells to different biological compartments is based primarily on differences defined using in vitro assays. Sensitivity to specific regulatory molecules and colony growth characteristics have provided the most sensitive markers to define different populations of progenitor cells using in vitro assays.

Indirect proof of the existence of cells more immature than the proerythroblast, the first recognizable cell of the erythropoietic lineage, was initially obtained by the measurement of ^{59}Fe labelled red blood cells released into the circulation 2-3 days after the injection of erythropoietin (Ep) into polycythemic mice, (where the recognizable erythroblastic compartment is suppressed), thus demonstrating the existence of an erythropoietin-responsive population (29). Similar short term responses can be obtained in liquid cultures (30). However, the in vitro ^{59}Fe incorporation assays measure the response of a more mature population than that present in polycythemic mice (31). Moreover, bulk isotope incorporation methods do not allow precise measurement of the capacity for proliferation and differentiation of individual cells.

The use of clonal analysis techniques to identify and characterize hematopoietic progenitor cells followed the development of suitable in vitro and in

vivo strategies. In general, two kinds of systems have been used: (1) those where a genetically determined clonal marker, e.g., a unique chromosomal change is available so that different types of dispersed clonal derivatives can be identified, and (2) those where the clonal progeny of a single precursor are obtained in close physical proximity to one another and in isolation from other cells or clones.

The first type of approach was first used in the 1960's, to demonstrate the distribution of marked cells in regenerating hemopoietic tissues (spleen, bone marrow and lymphoid organs) of sublethally irradiated animals, or lethally irradiated animals transplanted with cells carrying radiation-induced chromosomal changes (5,7). However, these methods were too cumbersome to allow further characterization of the stem cells capable of this regenerative response.

The second approach was first used in an in vivo context and has been accepted until recently (32) as an assay for murine hematopoietic stem cells. This assay is based on the formation of macroscopic mixed colonies in the spleens of irradiated recipients animals 8-14 days after the injection of histocompatible donor hematopoietic cells (33). More recent studies have now shown that most of the colonies seen after 7-8 days are transient and derived from cells (spleen colony-forming units or CFU-S) with limited proliferative potential (32). Pluripotent stem cells with extensive self-renewal capacity give rise to colonies in the spleen that may not become macroscopic until later, i.e., after 10 days post-transplantation.

In vitro assays utilize semi-solid media containing appropriate nutrients and stimulants. Originally, these in vitro clonal cell culture techniques provided assays for committed progenitors limited in their differentiation capacity to one pathway (34-37). Whatever the substrate (agar, methylcellu-

lose, or plasma clot), the lineage followed could be determined from the visual appearance of the colony when it had completed its growth; i.e., from the morphology and arrangement of the differentiated cells present in it. The size a colony can achieve is usually correlated with the length of the latent period that precedes the production of recognizably differentiated progeny (38-39).

All hematopoietic progenitor colony assays are indirect, because they actually measure only the differentiated daughter cells produced. Nevertheless, the existence of several stages of hematopoietic progenitor cell differentiation on each pathway has emerged from studies of the progenitors of different types of colonies. For example, at least 3 different types of erythroid colonies appearing sequentially in vitro have been described in the human as well as in the murine system (39,40). The cells of origin differ in the proportion of their numbers that are normally in S-phase, in their physical properties (41), in their sensitivity to Ep and leukocyte-derived factors (40) and in their statistical proximity to the pluripotent stem cell compartment from which they are derived (39). The largest human erythroid colonies contain more than 8 clusters of erythroblasts ($>10^3$ cells) by day 18 (i.e., have a proliferative capacity of at least 8-10 divisions). These large multiclustered ("burst") colonies originate from an immature erythroid progenitor (primitive burst forming unit-erythroid, or BFU-E) close to the CFU-S. A more differentiated cell type (the colony-forming unit-erythroid, or CFU-E), gives rise, after 3-4 divisions, to a single tight cluster of no more than 50-100 cells after 7-9 days. Intermediate between the primitive BFU-E and the CFU-E, are mature BFU-E that give rise to small bursts containing 3-8 clusters of erythroblasts (Figure 2A)(42).

Heterogeneity is also evident among progenitor cells committed to differentiate along the granulocytic-macrophage pathway. (This progenitor was

originally referred to as a colony-forming unit-culture, or CFU-C, but is now also commonly referred to as a colony-forming unit-granulocyte/macrophage, or CFU-GM (Figure 2B)). Here too a similar pattern has evolved, although greater flexibility in the proliferative activity expressed by individual granulopoietic progenitors (according to the concentration and type of colony-stimulating factors present (43)), makes their routine compartmentalization difficult. Three sub-populations have been described recently in the mouse. All three are bipotential, (i.e., capable of generating both granulocytes and macrophages), but differ in their buoyant densities, proliferative capacities, and cell cycle status in normal marrow. In addition, they require different stimuli for colony formation (44).

In vitro assay systems that support the proliferation and differentiation of pluripotent hemopoietic stem cells have been developed more recently. The first multilineage colonies to be described were grown in agar from mouse fetal liver cells stimulated by a factor (now known to be IL-3 -45) released by pokeweed mitogen activated spleen cells (46). Subsequently, similar colonies containing cells of the granulocyte, erythrocyte, macrophage and megakaryocytic lineages have been derived from adult mouse tissues. When these colonies achieve macroscopic size, the cell of origin (the CFU-GEMM) may overlap with a proportion of the CFU-S population (47), and may be capable of some self-renewal in vitro (48).

Multilineage colonies derived from single hematopoietic progenitor cells in human blood and marrow can be obtained in semi-solid cultures containing a factor(s) present in medium conditioned by PHA-stimulated human leukocytes and Ep (Figure 2C) (49,50). The presence of E-rosette positive, OKT4 positive cells and u-positive cells has recently been reported in some of these human multilineage colonies (51,52), suggesting that differentiation of both T and B lymphocytes from a common lympho-myeloid stem cell can occur in such cultures.

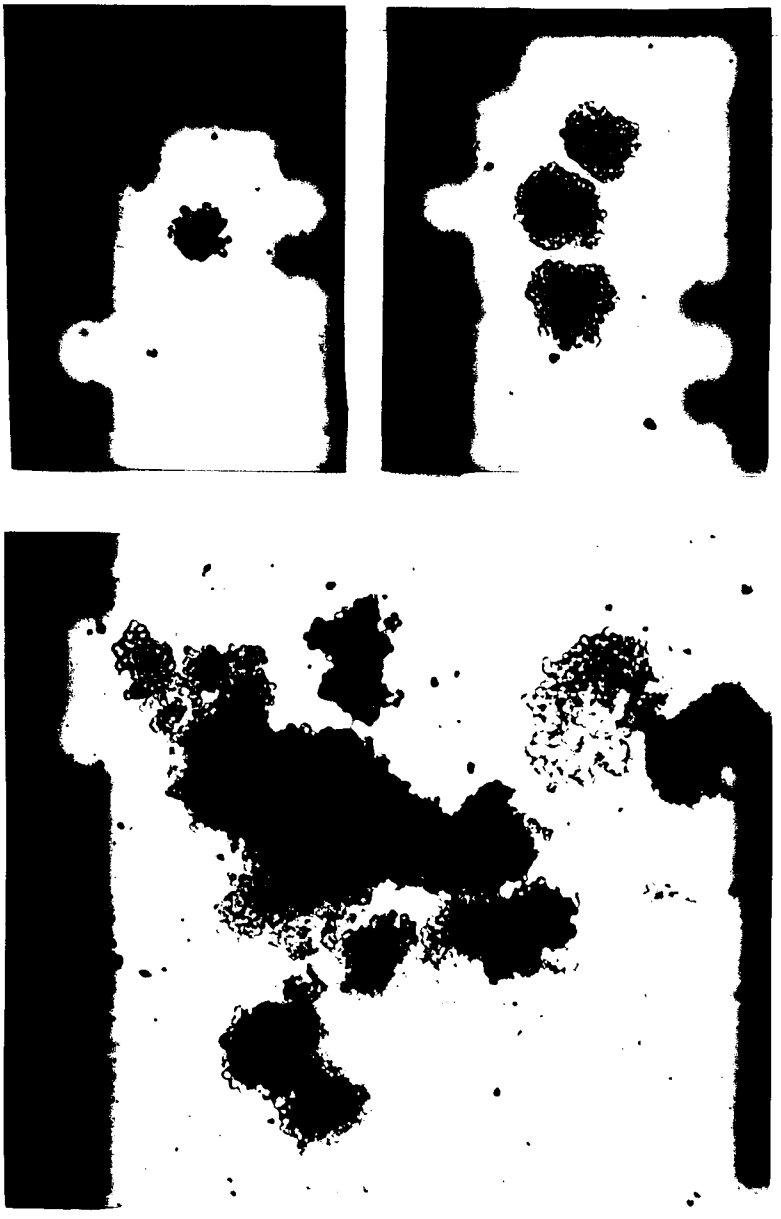

FIGURE 2. Different types of colonies generated by hematopoietic progenitors in methylcellulose assays (A) Stages of differentiation along the erythroid pathway. A single cluster from CFU-E, a 3-8 cluster-colony from a mature BFU-E, a > 8 cluster-colony from a primitive BFU-E (x100)

FIGURE 2. (B) Mixed erythroid-granulocytic colony from a CFU-G/E, (C) Granulocytic colony from a CFU-C (X80)

The clonal origin of these mixed colonies has been strengthened by G6PD analysis of the expanded T lymphocyte component of colonies derived originally from normal G6PD heterozygote women (53). However, the single cell origin of colonies containing apparently u-positive cells has not been conclusively established. Replating experiments have not been successful beyond the second generation transfer, and the self-renewal capacity demonstrable by these progenitor cells in vitro is relatively limited (54). At present, it is not clear whether this is due to their low intrinsic self-renewal capacity, or to the inadequacy of the culture conditions used to measure self-renewal. Studies of the proliferative state of human CFU-GEMM have shown them to be normally quiescent (55), similar to CFU-GEMM (56) and CFU-S (57) in mice.

A murine hematopoietic colony composed primarily of blasts on day 18 of culture has also been identified (58). Such colonies appear to be derived from a cell type that is more primitive than the CFU-GEMM, that begins to proliferate and differentiate much sooner after plating (59). The progenitor, or S-cell, possesses an extensive capacity for CFU-GEMM production as shown by blast colony replating experiments (58). A human equivalent has been found in umbilical cord blood but its presence in adult marrow or blood has not yet been documented (60). The existence of these late appearing blast colonies which contain large numbers of progenitors is due to the relative resistance of their progenitors to be stimulated into a cycling state for several days, in spite of the presence of a high concentration of stimulatory factors in the culture medium (59).

3) REGULATION OF STEM CELL FUNCTIONS

(A) Regulatory Molecules

During the life time of the individual, the number of circulating blood cells is maintained within a narrowly defined range. The precision of regulatory mechanisms must be considered remarkable, in view of the very short half-life of leukocytes and platelets (three and eight days respectively). The hematopoietic system also possesses the ability to respond rapidly to a variety of insults which requires enhanced production of mature cells. Regulatory mechanisms at the stem cell level in the marrow appear to depend on cell-cell interactions with local elements although long range and short range acting free molecules may also be involved. The complexity of stimulating and inhibiting serum activities interacting with specific progenitor cells is only beginning to be clarified. Most of these activities have been defined by their actions in in vitro systems. For example, as indicated above, the clonal growth in vitro of several hematopoietic progenitor cell types is dependent on the presence of molecules that demonstrate some lineage specificity.

Granulocyte-Macrophage-Colony-Stimulating Factors (the CSF's). Such factors were the first to be recognized by their ability to support the formation of granulocyte-macrophage colonies. A number of different molecular species have been demonstrated to have this property and in each case colony formation is completely dependent on continuous stimulation by the factor. The various CSF's differ in molecular weight, carbohydrate content, in their ability to support complete differentiation along various pathways, and in the cell type from which they are usually obtained (61). Although it is clear that many of these factors are produced in vivo, their role in the physiological regulation of granulocyte and macrophage production is still not established. For example, G-CSF active in the mouse has been isolated from the myelomono-

cytic cell line WEHI-3 (62), and post-endotoxin serum (63); GM-CSF from lung tissue (64), and PWM-stimulated spleen cells (65); M-CSF from L-cell (66), and fetal tissue (67). Very recently, two of these, G-CSF and GM-CSF, have been molecularly cloned and shown to be dinstinct gene products (68,69). For stimulation of human granulocyte-macrophage colony formation in culture, the usual sources of GM-CSF are activated leukocyte-conditioned medium (70), or placental conditioned medium (71). Certain tumor cell lines have also now been shown to release a factor with this activity (72).

A major source of GM-CSF in vivo may be a subpopulation of phagocytic mononuclear cells (73), although GM-CSF may be synthezised by other cell types (61). Other factors have also been implicated in the regulation of granulopoiesis; for example, interferon and prostaglandin, both of which may be secreted by certain types of activated monocytes, can modify the responsiveness of granulopoietic progenitors to GM-CSF and are potent inhibitors of CSF-induced monocyte proliferation (74,76). Another inhibitory molecule that has been isolated from the mature granulocyte population is lactoferrin (77).

Erythroid Colony-stimulating Factors (Erythropoietin and Interleukin-3). A similar complexity of humoral regulators has been shown to act on cells progressing along the erythroid pathway. These include Ep and a single factor alternatively referred to as burst-promoting activity (BPA) (78), Interleukin-3 (IL-3) (79), hematopoietic cell growth factor (HCGF) (80), P-cell growth factor (81), and multi-CSF (82). The precise action of Ep or IL-3 in vitro has not been clearly elucidated and their mechanisms of action are still largely unknown (83). Until recently, the lack of purified molecules hampered the development of reproducible and specific assays. However, both Ep (84), and IL-3 (85-86), like G-CSF and GM-CSF, have now been molecularly cloned and more rapid progress may be anticipated. Nevertheless, it was already clear from

earlier studies that the sensitivity of erythroid precursors to Ep increases as the cells differentiate. Concomitantly these may become less requiring of the presence of IL-3 (87). Immature BFU-E are not detected when IL-3 concentrations are low (IL-3 is usually provided by addition of media conditioned by agar or lectin-stimulated leukocyte-rich fractions of human blood for human progenitors, and by media conditioned by pokeweed-stimulated mouse spleen cells for mouse progenitors). IL-3 does not appear to be a strict requirement for CFU-E, and mature BFU-E behave as an intermediate population (42,87,88). Ep has been shown to be a compulsory factor for late erythroid maturation under at least some conditions but, at this level, it is difficult to distinguish whether it acts as a survival factor, or whether it may also have mitogenic activity. At early stages of erythroid cell differentiation, evidence both for (89), and against (90), a mitogenic role of Ep has been published. In contrast to GM-CSF, Ep has clearly been demonstrated to be the physiological stimulus of the erythroid pathway in vivo (91). A sequential requirement for several growth factors has also been described for differentiating megakaryocytic progenitors (92). Inhibitory molecules affecting erythropoiesis have been described but, as for granulopoietic inhibitors, their significance in vivo remains obscure.

Early on, assessment of factors that support the growth of various types of colonies led to the idea that most represented lineage-specific growth factors each playing a major role only after lineage restriction had occurred. On the other hand, IL-3 containing preparations were known to be capable of stimulating a variety of both uncommitted and committed cell types (93), and this has since been confirmed with homogeneous preparations (94). It has also been necessary to generalize this latter concept to other factors previously thought to possess lineage specificity. For example both GM-CSF (95) and G-CSF

(96) have been shown to be able to sustain the first few divisions of murine pluripotent precursors, although neither M-CSF nor Ep were able to do so (97,98).

In addition to humoral regulators that act on committed progenitors, short and long-range stimulatory and inhibitory materials that act on CFU-S proliferation have also been studied (99). As just indicated, IL-3 (98), GM-CSF (95), and G-CSF (96), can all do this, although in the latter instance the response is not sustained. The relevance of either of these molecules to mechanisms that activate resting stem cells after treatments such as irradiation or chemotherapy is unknown (100,101).

(B) Regulatory Role of the Microenvironment

Interactions between supportive structures and effector cells, of different embryonic origin, that are essential for tissue maturation and the acquisition of specialized functions have been described in many organs. In the thymus for example, the acquisition by migrating blood borne cells of the phenotypic and functional characteristics of thymocytes is thought to occur through successive interactions with specific components of the thymic microenvironment (102). The precise nature of these interactions is largely unknown, but it is likely that responses to known stimulators as well as close range cell-cell interactions are involved. Since harmonious development implies well-ordered interactions, abnormalities in tissue organization have been regarded as a possible contributing or characteristic factor in the evolution of a malignant population. Conversely, it has been shown that malignancy may sometimes be reversed under the influence of appropriate microenvironmental control mechanisms. Thus, teratocarcinoma cells injected in syngeneic blastocysts participate in the development of normal mosaic mice, free of tumors

(103,104). Similarly, myeloid leukemic cells transplanted into embryos can participate in normal hemopoietic differentiation (105). This potential to modulate a cell's phenotype from malignant to normal by external factors in some circumstances offers an important possible alternative to cytotoxic therapy in the hematopoietic malignancies, particularly those where the execution of aberrant differentiation programs is a major clinical problem (106).

Experimental Arguments for Stromal-hematopoietic Interactions. Before experimental evidence was obtained to indicate a functional role of non-circulating marrow components in regulating hematopoiesis, this was suggested by findings which showed an intimate structural association between hematopoietic and stromal cells in the marrow. Murine and human bone marrow architecture has now been extensively studied by electron microscopy. The striking feature is the absence of direct contact between maturing hemopoietic cells and the blood stream. Both compartments are separated by endothelial and "reticular cells" (107).

Endothelial cells completely cover the inner surface of the marrow sinuses and are lined on their outer surface (towards the marrow) by a basement membrane made of collagen IV (108). Marrow cell egress occurs through the cytoplasm of these endothelial cells and their endocytic properties allow blood nutrients to reach the hemopoietic spaces. Cells described morphologically as adventitial reticular cells are located between the sinus wall and the hematopoietic spaces and are characterized by extensive cytoplasmic processes which appear to provide the physical support for developing cells. Whether or not these cells represent the major fibroblast population of the marrow is still debated, as it has been reported that fibroblasts can originate from large blood vessels or from bone itself (109).

A distinct embryonic origin of stromal and hematopoietic cells is supported by numerous data. Fibroblasts are thought to have a mesenchymal origin whereas hematopoietic cells are believed to originate from cells in the yolk sac blood islands (110). In addition, G6PD and chromosome studies have shown that marrow fibroblasts are not part of the neoplastic clone in several such disorders (111,112). However, recent studies have suggested that some stromal subpopulations may have a common origin with hematopoietic cells (113-115).

Observations during mouse fetal development, stem cell engraftment after irradiation (116), or marrow depletion support the concept of a necessary stromal seeding space as a prerequisite for hematopoiesis to take place (117). However, this too has recently been contested (118). Conditions required for the development of hematopoiesis have been defined by establishing ectopic areas of hematopoiesis (119,120). The classical non-transplantability of hemopoietic stromal cells by the intra-venous route (121) has also recently been contested (122,123), and their ability to migrate into suitable inductive environments confirmed (124). However, the donor origin of marrow fibroblasts in patients following allogeneic bone marrow transplantation has not been confirmed (125-127), although such studies do not exclude the possibility that some stromal elements may be donor-derived (128).

Characterization of Stromal Cells In Vitro. Attempts to define properties of stromal cell precursors using short term colony assays have not been as successful as for hematopoietic precursors. This can be partly explained by the low numbers of stromal precursors in available specimens (aspirates), the lack of selective methods to separate them from the hematopoietic component and the relative lack of unique stromal cell markers, either morphological or functional. The fibroblast precursor cell (CFU-F), is the only one whose

properties have been extensively studied even though their number in fresh human marrow is highly variable, ranging from 15 to 60 per 10^6 cells (129). The cells are known to be adherent, trypsin-sensitive, light-density, non-phagocytic and non-cycling (130-132). Precursors of other stromal cell types may exist but have not been identified (133). Vascular endothelial cells are currently grown in primary monolayers (108), but the growth of capillary endothelial cells has only very recently been reported (134).

The identification of stromal cells is therefore presently limited to an analysis of their secretion products. Fibroblasts are believed to specifically secrete collagen I and III (135-136), whereas endothelial cells secrete collagen IV (108), Von Willebrand factor (VWF (137)), and laminin (133), a protein that mediates the attachment of cells to collagen IV. Both cell types secrete fibronectin, a major adhesive glycoprotein (138). All these proteins (except VWF), are components of the extracellular matrix (ECM), first defined in connective tissue. A growth promoting activity of the ECM has been demonstrated in other systems (139,140). Other components of the ECM are the glycosaminoglycans (GAG)(141). These coat most of the cells in the marrow and the proportions on each cell varies according to its state of differentiation (142). An influence of various GAG's on the regulation of CFU-S has recently been demonstrated in mouse long-term cultures (143).

Experimental data indicating a direct influence of stromal cells on hematopoietic cell differentiation are rare. Most of the designs have used the direct addition of ECM components or different cell types (mostly fibroblasts) to fresh marrow colony assays. Results are very controversial, and stimulatory as well as inhibitory effects have been observed on colony growth (131,132,144-146). One possible way for stromal cells to be effective is through the secretion of colony-stimulating factors (147,148). For example, it appears that

many of the constituents of the stromal population are able to produce GM-CSF, and several stromal cell lines are constitutive producers (149). CSF secretion by fibroblasts has also been documented (150). Vascular endothelial cells are able to produce CSF as well, and these are active in both murine and human non-adherent cell assays (151,152). Unfortunately, the identification of these stimulators in conditioned media does not help to establish whether these may be mediators of similar effects via direct cell-cell interactions in the marrow microenvironment.

Defective Interactions between Stroma and Hematopoiesis. The best known example of a microenvironmental defect is that characteristic of $S1/S1^{d}$ mice. These mice, who have a rare heterozygous allotype at the steel locus (S1) on chromosome 10, have abnormalities affecting melanocytes and germ cell development in addition to a macrocytic anemia (153). The hematopoietic defect is not cured by transplantation of histocompatible blood forming stem cells, but transplantation of intact hematopoietic stroma to this strain of mice can improve their anemia (154,155). A similar model of deficient hematopoiesis due to an abnormality in the microenvironment does not exist in humans and the data suggesting a possible analogous microenvironment defect in the genesis of aplastic anemia are not very convincing (156-159). ECM modifications and abnormal CFU-F numbers have been described in leukemic patients (131,132,160-162). However, the significance of these observations remains obscure. Another example of interactions between stroma and hematopoiesis is seen in secondary myelofibrosis characteristic of CML patients (10,163,164).

(C) Theories of Stem Cell Commitment

The generally accepted model of hematopoietic stem cell differentiation implies a structured hierarchy of progenitor cells in which movement from a

multipotential compartment to a more restricted and eventually a monopotential compartment is irreversible (Figure 1). Unequivocal proof of the existence of cells that are themselves pluripotent and that can also generate new pluripotent daughter cells (self-renewal) has been obtained as discussed above. Other features of the model have been supported by replating studies which have shown committed progenitors to be derived from pluripotent precursors in varying combinations but not the reverse (165). Ultimately, questions relating to the irreversibility of restriction of differentiation potential and proliferative capacity (and whether or how these processes may be linked) will have to be addressed at the DNA level, for example as has recently been possible for B lymphocyte differentiation (166,167). At present, however, the data base on which such an analysis would depend is insufficient to permit clear cut answers to be obtained. Thus, the models that exist are derived from retrospective and hence indirect studies of the spectra of clonally generated progenitor cells and their mature progeny.

Two theories of how stem cell self-renewal and differentiation is regulated have been proposed. The deterministic theory postulates that the behavior of a stem cell is simply a reflection of the directive influence of the stromal environment in which it is found. Such a model was based on the findings from sequential cytological analyses of spleen and marrow colonies. These showed that initially, colonies contained only one lineage of differentiation with multiple lineages within single colonies appearing later. The spleen was viewed as a composite of small, local acting lineage-inductive microenvironments, each dictating differentiation along a given line of hematopoietic differentiation (168). Mixed colonies were then thought to result from the growth and extension of the progeny of a single stem cell into several different microenvironments. On a larger scale, marrow stroma was found to

favour granulopoiesis whereas erythropoiesis was favoured in the spleen, thus providing some evidence that different microenvironments can have different effects (169). It was further suggested that the mechanism involved was to induce the stem cell into a state of hormone (e.g., Ep or GM-CSF) responsiveness (170). In fact, as described above, it has now been shown that pluripotent stem cells are already responsive to GM-CSF (98).

A derivative theory based on stem cell competition experiments gives a deterministic role to regulatory molecules such as GM-CSF and Ep (171). Recent experiments analyzing the response of paired daughters of mouse CFU-C incubated either in GM-CSF or M-CSF have shown that humoral regulators can at least under certain conditions influence the decision of cells to be committed towards either the granulocytic or macrophage lineage (172). It is therefore possible that other molecules may exert extrinsic influences on the probability of pluripotent cell commitment to other pathways.

The second theory focuses on the probabilistic nature of the process by which self-renewal capacity is lost. Loss of self-renewal is viewed as an event whose control appears to include a random component that is intrinsic to the stem cell; the role of external influences being either to modify the probability of commitment or to allow expression of the restricted potential of an already committed cell. This stochastic model has received much support from recent experiments. It was developed initially to explain the high variability observed in the number of CFU-S in individual spleen colonies in vivo without the need to invoke any influence of the microenvironment (173). The demonstration of a similar variability in the self-renewal of CFU-GEMM in individual macroscopic mixed colonies produced in vitro provided strong evidence in favor of self-renewal determination having a stochastic component (48). More recently, this finding has been repeated in analyses of multi-

lineage colonies generated in cultures seeded each with a single pluripotent cell (174-176). Not surprisingly, the two models are not mutually exclusive, as long as it is assumed that the probabilities of stem cell self-renewal and restriction of differentiation potential are not necessarily fixed, but perhaps, as discussed above, subject to both intrinsically and extrinsically controlled factors. It therefore seems likely that elements of both models will prove to be correct, and the possible mechanisms of influencing stem cell behavior will prove more complex than initially anticipated.

4) LONG-TERM BONE MARROW CULTURES

(A) Description of the System

The successful maintenance of hematopoietic stem cells in culture for extensive periods was first described by Dexter et al in 1977. The primary feature of this so-named "long-term marrow culture system" is the apparent dependency of stem cell maintenance on the presence of other cells that form a complex adherent layer in the culture. This layer develops as a result of the proliferation of stromal precursors present in the marrow sample, and is thought to serve as the in vitro equivalent of the hematopoietic microenvironment (177,178).

Above the adherent layer, and in constant exchange with it, is a non-adherent cell population which consists almost exclusively of hematopoietic cells (179). The cultures are maintained by regular replacement of part (or all) of the nutrient medium which contains serum (horse and/or fetal calf) and usually also hydrocortisone (180). However, none of the stimulating molecules required in semi-solid assays are exogenously provided. The behaviour of the cells maintained in this system can be readily assessed by monitoring the progenitor content of the non-adherent cell fraction at weekly intervals using

the various colony assays described above. In long-term mouse marrow cultures, CFU-S and granulopoiesis can be maintained for at least 30 weeks (181,182), and precursor cells removed appear to have similar characteristics and functions to cell populations found in fresh marrow (183).

The culture system regularly supports granulocyte maturation up to the mature neutrophils and macrophages. On the other hand, morphologically recognizable erythroid cells and eosinophils are never found unless culture conditions are specifically modified to allow their production to occur. Similarly, CFU-E and mature BFU-E are not detected in supernatant fractions (181,185). Immature (pluripotent) BFU-E are, however, maintained in such cultures (185-187). The "block" in erythropoietic differentiation can be partially overcome by adding erythropoietin, this leads to the production of some CFU-E. Maturation to the hemoglobin-producing stage requires the addition of either a mechanical stimulus or anemic mouse serum (188,189).

Primitive precursors capable of B and T cell production in vivo are also maintained in long-term bone marrow cultures but their differentiation into mature lymphocytes in the cultures is also blocked (190,191). However, altering cultures conditions (replacement of horse serum by a low concentration of fetal calf serum and elimination of the presence of corticosteroids) can allow normal murine B cells and their precursors, including pre-B cells with unrearranged immunoglobulin genes, to be maintained for periods of 20 weeks or more (192,193).

(B) The Role of the Adherent Layer in Long-term Bone Marrow Culture Composition. The adherent layer of long-term marrow cultures contains a variety of cell types that have not yet been fully characterized (194). Three main types were initially described in mouse cultures on the basis of electron

microscopy. These were large, fat-containing cells, flattened endothelial-like cells, and macrophages (195). In human cultures, similar fat-laden cells and macrophages as well as fibroblasts have also been generally observed (196). The presence of endothelial cells is still controversial (113,197,198). However, in spite of the agreement on the presence of some cell types, their spatial organization and its possible significance for stem cell maintenance are still not known (199-201). More recently many of the secretion products of the adherent cells have been studied. Many of these are normal extra cellular matrix (ECM) components, e.g.,laminin, type IV collagen and VWF factor, type I and III collagen and fibronectin. Their presence is thought to indicate the activity of different cell types, e.g., the first 3 being derived from endothelial cells, the latter 3 from fibroblasts. It has also been shown that these molecules are deposited in an orderly sequence during the formation of the adherent layer (202). It seems likely that the ECM offers not only a supportive adhesive structure, but may also play a role in the regulation of hematopoiesis in vitro as has been shown in other systems. Modulation of the synthesis of proteoglycans, another component of the ECM long-term cultures, has recently been shown to influence CFU-S proliferation (143).

Besides the stromal component, the adherent layer also contains hematopoietic cells. Most of these are either macrophages or very early cells of the granulocytic series cells, although many types of early progenitor cells are also found in the adherent layer (181,196,203, see also Chapter II).

CFU-S are consistently detected in the adherent layer of mouse cultures for several months, and the cumulative yield of CFU-S removed from cultures provides a ready indication that this population is amplified. Similarly, continual repopulation of the non-adherent fraction with weekly removal of all the non-adherent cells has shown that the non-adherent fraction is maintained by release of cells from the adherent layer (182).

Several studies have established that stem cells located within the adherent layer have different properties than those found in the non-adherent fraction. CFU-S in the adherent layer of 6 day-old cultures have a higher self-renewal capacity than the non-adherent CFU-S and this discrepancy is maintained thereafter (204). Similarly, the ability of adherent cells to reconstitute myeloid and lymphoid systems of irradiated recipients is clearly higher than that of non-adherent cells (205).

What determines such discrepancies is not known. However, regulatory molecules have been shown to be produced by stromal cells that regulate the proliferative activity of CFU-S, but not CFU-C, according to a cycling pattern (206). Granulocyte-macrophage colony-stimulating factors are usually not demonstrable in long-term marrow cultures (177) although this apparent absence of CSF has recently been shown to be due to local consumption (207,208), and measurable CSF levels have been obtained using radioimmunoassays (209). The fact that purified M-CSF or antibodies against M-CSF do not modify the granulopoietic pattern suggests that this factor may not be of major importance but does not rule out a role for GM-CSF or G-CSF (210,211). Neither IL-3 nor Ep have been detected in biological assays of long-term culture supernatants (212), although adherent cells from mouse long-term cultures exhibit greater IL-3 activity than normal marrow cells (212). However, none of these findings bear on the possibility that such molecules may mediate stimulatory effects via direct contact of progenitors with stromal elements in the adherent layer (213).

Reproduction of Microenvironmental Defects in the Long-term Marrow Culture System. Maintenance of hematopoiesis does not occur in long-term marrow cultures if the formation of the marrow-derived adherent layer is inhibited or if it is defective (214,215). The first situation results when marrow cells

are added to siliconized flasks, or if the adherent cells are quantitatively or qualitatively deficient. Such a deficiency is apparent when cultures are established from irradiated mice (4 X 450 Rds), even though the adherent layer may appear to be morphologically and quantitatively normal (216,217). A similar result is obtained when cultures are initiated with marrow cells from $S1/S1^d$ mice. However, $S1/S1^d$ stem cells can be maintained if $S1/S1^d$ cells are cultured on wild-type (+/+) adherent cells or on adherent cells derived from W/W^v mice whose stem cells are defective (218). These experiments provide support for the view that the stem cell maintenance in long-term marrow cultures is regulated by the same mechanisms that operate in the marrow microenvironment in vivo.

5) NEOPLASTIC DISORDERS OF THE STEM CELL: CML AND AML.

(A) The Myeloproliferative Disorders and the Acute Leukemias: Overall View

Human neoplastic disorders of the myeloid hematopoietic system include two groups: the acute leukemias and the myeloproliferative disorders that are characterized by a chronic phase but may also terminate in an acute leukemia. Separation of these disorders in this way is based on historically evolved clinical findings. However, as our understanding of the underlying cell biology and molecular genetics of these diseases increases, it is recognized that new categories may well emerge. The myeloproliferative disorders classically include chronic myelogenous leukemia (CML), essential thrombocythemia (ET), polycythemia vera (PV), and idiopathic myelofibrosis with myeloid metaplasia (MF). All are characterized by hyperplasia of all three myeloid compartments on examination of core biopsies of the bone marrow even though differentiated circulating cells of only one lineage my appear in excess in the peripheral blood (i.e., RBC in PV, PMN in CML, platelets in ET). Acute

myeloid leukemia (AML), has been considered as a separate entity (or entities) because abnormal cell proliferation most commonly seemed to be associated with the granulocytic pathway and involved the accumulation of excess numbers of very immature appearing cells, therefore called blasts. This excessive production of non-functional myeloid cells, usually with some granulopoietic features was considered to represent a situation that contrasted with that typical of the myeloproliferative disorders where a large population of fully differentiated cells is produced (219).

Typically, the myeloproliferative disorders are characterized by a rather slow course where a mildly compromising chronic steady state can often seem to be maintained for several years with minimal chemotherapy. A diagnosis of CML is usually suggested in a patient still asymptomatic by the detection of a slightly elevated leukocyte count (15,000-30,000 PMN per mm^3) and circulating immature members of the granulocyte series. Diagnosis is definitive if splenomegaly, marrow hypercellularity and the characteristic Philadelphia chromosome (Ph^1) are present (220).

In contrast, the acute leukemias are usually much more explosive at presentation. This is due to the marrow insufficiency created by an apparent complete replacement of normal cells by non-functional blasts and their precursors, and their inhibitory action on residual normal progenitors. However, in some instances, mostly in older patients, a "preleukemic" phase with predominant features of chronic marrow insufficiency can be observed to precede a picture of overt AML (221).

Even though CML at first appears less aggressive than AML, its prognosis is still fatal and this situation has not been improved by recent chemotherapeutic trials. The natural course of the disease leads on average in 30-40 months to blast crisis which, simply stated, represents the conversion of CML

into an AML-like picture (60% of cases) or ALL-like picture (30% of cases) (220) recently shown to be of a B-cell or pre-B cell type (222). Blast crisis is viewed as the result of a process referred to as clonal evolution, a process whereby a cell within the malignant clone undergoes further genetic alteration, outgrows the members of the original clone and shows a decreased ability to mature normally (223). Typically such sub-populations do not respond well to chemotherapy and re-induction of chronic phase is obtained rarely and usually only when an ALL type blast crisis has evolved.

Induction of a complete remission by aggressive chemotherapy can usually be successful in the majority of patients with AML under 80 years although the restoration of normal hemopoiesis is relatively short-lived (224). This is because total eradication of the leukemic stem cell population is not readily achieved with current chemotherapeutic regimens and relapse is therefore eventually inevitable. Cure of AML is thus at the present time an unrealistic expectation unless rescue by matched sibling allogeneic bone marrow transplantation is an option. In this case higher doses of chemotherapy and total body irradiation can be used effectively (225). In CML allogeneic bone marrow transplantation has also produced long-lasting elimination of the Ph^1-positive clone, although results are poor unless the transplant is given in the chronic phase of the disease (226-228).

CML and AML are both clonal disorders, i.e., they originate in a cell which acquires such a proliferative advantage over all other hematopoietic cells present that by the time of diagnosis, most of the cells present in the marrow or blood are progeny of that initially transformed cell.

(B) Chronic Myelogenous Leukemia (CML)

Origin in a Pluripotent Stem Cell. Identification of cells belonging to a single clone depends on the availability of genetically determined markers that allow clonal and non-clonal cells to be distinguished. The most commonly used marker for this purpose has been the X-linked gene for G6PD (glucose-6-phosphate dehydrogenase) and specific chromosomal abnormalities acquired early on in the development of the neoplastic clone. The enzyme G6PD has several alleles, and some of these specify isoenzymes that can be electrophoretically distinguished. Since random inactivation of one chromosome X occurs in each cell of every female early on in embryonic development, all females who are heterozygous at this locus are mosaics with respect to their G6PD phenotype. Usually this results in a balanced mosaicism with a 50:50 ratio of expression of the two isoenzymes in a given tissue or cell type. Thus, if only one isoenzyme is detected, it is reasonable to assume that the population originated from a single cell with a relative proliferative advantage (229).

Similarly, a unique chromosomal marker may be used to identify cells belonging to the same clone. Both methods have been used to determine the origin of the hematopoietic cells present in patients with AML or CML. G6PD isoenzyme studies and chromosomal findings are complementary but not strictly identical. The latter visualizes an aberration that is acquired during the neoplastic process and its detection is possible only in proliferating cells. Moreover, it is not always possible to know at what stage in the development of the leukemic clone it first appeared and it is likely that many chromosomal changes are not early events (230). As a result, cytogenetic analysis cannot be used with confidence to definitively identify non-leukemic cells. The use of G6PD depends on the expression of a normal allele to delineate the origin of a given cell regardless of its stage of differentiation but interpretation relies on a number of statistical assumptions.

In 95% of patients with CML in chronic phase, bone marrow cells show an abnormal karyotype (231). The t(9,22) translocation is the most frequent: this translocation is non-constitutive, reciprocal, and balanced. A piece of the long arm of chromosome 9 is translocated to the long arm of chromosome 22 and usually, but not always, the opposite occurs (232). The resultant shorter chromosome 22 is called the Philadelphia or Ph^1 chromosome (233). Because of the consistency of the translocation of the c-abl containing region of chromosome 9 to a consistent breakpoint region on chromosome 22, it has been suggested that c-abl may be involved in the pathogenesis of CML (234).

Chromosomal abnormalities are generally encountered in 50% of AML patients using conventional banding techniques (235,236). Recent studies using more sensitive methods have indicated that some cells from all AML patients exhibit detectable chromosomal abnormalities (237). In AML also, the chromosomal abnormalities observed are restricted to cells of hematopoietic origin.

G6PD isoenzyme and cytogenetic analyses have been used to determine not only whether a disorder is clonal or not but also to obtain information about the type of progenitor that initially acquired a selective growth advantage. Because all peripheral blood and bone marrow cells (i.e., granulocytes, erythroblasts, and megakaryocytes or platelets) display only one isoenzyme pattern in CML (238), it has been concluded that the disease originates in a single member of the pluripotent stem cell compartment although initially, more than one cell type may have been altered. Similar conclusions have been obtained from cytogenetic studies where early on the Ph^1-chromosome was shown to be present in bone marrow cells differentiating along all 3 myeloid lineages (239). These findings have now been confirmed by recent data at the precursor level: cells from erythroid and multilineage colonies as well as from granulocytic colonies generated in vitro from CML marrow progenitors (CFU-E, BFU-E,

CFU-C) generally have the Ph^1-chromosome (240-242), and display a single G6PD isoenzyme pattern in heterozygotic women with CML (243). Similar findings in mixed colonies have given a direct confirmation of myeloid pluripotent stem cell involvement (CFU-GEMM) (244,245).

Some but not all lymphocytes are also commonly generated from clonal (i.e., neoplastic) progenitors. Strong arguments exist for the involvement of the B cell lineage. These included the direct demonstration of the Ph^1-chromosome in B cells from most CML patients (246), the detection of a single isoenzyme pattern of the non-rosetting (B-) lymphocyte fraction, and the isolation from a G6PD heterozygote of an unexpectedly high number of EBV-transformed B cell lines that expressed the same G6PD isoenzyme as the malignant clone (but were Ph^1-negative) (230). Finally, the recent demonstration of the pre-B genotype of a significant proportion of lymphoid blast crises (222), favors the concept that the original stem cell transformed is usually one capable of producing B lymphocytes as well as all of the myeloid cell types. Origin of T lymphocytes in CML is much more controversial. In general, E-rosetting cells are Ph^1-negative as are PHA-stimulated blood lymphocytes (247). Both are polyclonal according to G6PD isoenzyme patterns(248). However, there has been some discordance between results pertaining to the chronic versus more poorly controlled later phases of CML. In the latter situation, it has been reported that all cells in the "T lymphocyte fraction" belong to the neoplastic clone (248). It is not yet resolved whether the persisting non-clonal T lymphocytes represent long-lived cells, whose time of first appearance antedated the neoplastic event, or whether they may arise from an active T cell restricted stem cell, not involved in the CML clone.

Differentiation Potential of CML Stem Cells. In CML, as in PV, the growth advantage acquired by the transformed stem cells does not appear to affect the overall differentiation process. During the chronic phase, clonally derived committed precursors of each hematopoietic lineage are capable of terminal differentiation and give rise to mature and functional granulocytes, monocytes, red cells and platelets in vivo. This is also seen when Ph^1-positive progenitors are assayed in semi-solid culture systems, where they give rise to colonies that are phenotypically undistinguishable from colonies derived from nonclonal progenitors (242).

In vitro studies of the early progenitor cell compartments in patients with CML have shown that these are grossly enlarged by clonal members. Measurements of circulating CFU-E, BFU-E, CFU-C, CFU-M (colony-forming unit-megakaryocyte) and CFU-GEMM numbers have shown these to be increased up to several thousand fold, with the number increasing approximately as the square of the WBC count (249-252). This strongly suggests that the numbers of these progenitors is also likely to be significantly increased in the marrow. However, their concentration in the marrow is not increased (249), so that any absolute increase would only be related to the increased volume of hematopoietic tissue in the marrow cavities. Both karyotype and G6PD isoenzyme studies of single colonies have shown that these increases in progenitor cell numbers represent an expansion of the abnormal clone.

Comparison of the size of various committed progenitor compartments (e.g., CFU-C versus BFU-E) in individual CML patients where most (>90%) of the progenitors are clonal derivatives, has shown that the relative numbers of cells progressing down the initial part of each myeloid pathway is not on average grossly altered from the norm (253). This suggests that the mechanisms that underly the restriction of stem cell differentiation potential are not affected

in spite of the dramatic amplification of their numbers. The high increase in primitive granulopoietic progenitors is not unexpected in view of the large increase in circulating granulocytes. However, the corresponding large increase in CFU-E numbers is difficult to reconcile with the anemia usually seen in CML patients and indicates that terminal erythropoietic differentiation is adversely affected, either due to an intrinsic alteration in the cells themselves, or because of secondary effects of the disease (e.g., marrow crowding). Erythropoietic progenitors in many CML patients show Ep-independent erythroid differentiation in vitro (254), suggesting some intrinsic alterations to these cells. Thus, the ability to execute completely normal differentiation programs may be less clear-cut than originally believed.

Does Residual Normal Hematopoiesis Continue in CML Patients? At the time when this study was initiated, the question of whether residual normal stem cells persist or not during the chronic phase of CML was controversial. G6PD isoenzyme studies had failed to detect residual normal cells either in the CFU-C compartment (243), or in more mature progeny (10). It was known that occasional Ph^1-negative metaphases could be found in direct marrow preparations in about 30% of the patients at early stages of their disease, but the possibility that such metaphases simply represent rare dividing non-hematopoietic cells (e.g., fibroblasts) or T cells could not be ruled out (255). More recently, the consistent conversion of a Ph^1-positive marrow chromosomal pattern to a mosaic pattern after aggressive chemotherapy provided strong evidence that normal stem cells are present in these patients, at least early on in their disease, although it was then necessary to postulate that their expression was inhibited by the presence of the neoplastic clone (256,257). The nonclonal origin of these Ph^1-negative hematopoietic cells was indicated in one case where return of both G6PD isoenzyme types was demonstrated (258).

However, it is usually not possible to obtain as complete or prolonged a reversion to normalcy as is typical of AML patients in remission.

The first data using karyotype analysis of CML stem cells performed on single or pooled granulocytic colonies regularly failed to demonstrate Ph^1-negative cells except in one study (259). Absence of detectable numbers of persisting normal committed progenitors in CML differed from the situation in PV, another clonal stem cell disorder, where some nonclonal early precursors were regularly found (260). However, an explanation for this apparent discrepancy can be found if the relative sizes of the progenitor compartments in CML and PV are taken into consideration. In PV, these are not commonly significantly increased and therefore, detection of a residual normal compartment is feasible (261). However, since the progenitor compartments in most CML patients are increased more than 10-fold (at least in the blood), even an unaltered residual normal component would be diluted to almost undetectable levels. It is therefore not surprising that Ph^1-negative progenitors have rarely been found in CML patients. Moreover, in very recent studies, it has been shown that in those occasional CML patients whose progenitor numbers were not yet grossly elevated (WBC<30,000 per mm^3), Ph^1-negative progenitors could be consistently detected (245).

Regulatory Abnormalities. Defective PGE_1 inhibition has been suggested as a factor contributing to the excess myelopoiesis seen in CML as well as in other myeloproliferative disorders (262). It has been reported that concentrations of PGE_1 that inhibit normal CFU-C colony formation do not inhibit CFU-C from CML patients, either because the latter are less sensitive to PGE_1 or are abnormal in their ability to circumvent PGE_1 inhibition in the presence of CSF concentrations that are ineffective on normal CFU-C. Reduced lactoferrin secretion by mature CML granulocytes and some degree of insensitivity of CML

monocytes to lactoferrin has also been described, suggesting other possible mechanisms of faulty negative feedback regulation (263). However, these findings have not been confirmed and the significance of these observations has not yet been established.

Among other mechanisms possibly involved in the neoplastic growth characteristic of the CML clone is one that prevents the neoplastic cells from entering G_o (264). Older studies indicated near normal labelling indices for morphologically recognizable precursors and suggested that the hyperplasia seen in CML must therefore be explained by changes in earlier compartments (265). As indicated above, it is now known that all progenitor numbers appear to be increased. Recent experiments with suicide techniques have further revealed that most of the early progenitors are cycling, including the primitive BFU-E and CFU-GEMM (266,267), whereas in normal individuals, very few of these progenitors are in cycle.

If these anomalies are indeed primary to the disease process, it is of interest to consider whether the t(9,22) translocation is directly responsible. With the recent demonstration that the translocation of the cellular proto-oncogene c-abl (normally on the distal portion of the long arm of chromosome 9) to a specific region on chromosome 22 (268) is a consistent feature of Ph^1-positive CML, including those involving more complex translocations (269), it has been suggested that altered expression of c-abl may be of primary importance. This has been given further weight by the recent demonstration of an altered c-abl message in Ph^1-positive cells (270,271).

(C) Acute Myelogenous Leukemia (AML)

Classification. AML has been recognized for a long time as a very heterogenous group of disorders. Cytology and cytochemistry methods are currently used to recognize at least 5 subgroups the criteria for which are given by the FAB classification (272). Similar heterogeneity is encountered in the types of chromosomal abnormalities seen, many of which have now been shown to be non-random. Some of these show correlations with the morphological classification (273). For example, the t(15, 17) in M3 promyelocytic leukemia (274), the t(8,21) in M2 (myeloblastic) leukemia (275), and changes to chromosome 16 in an M4 subtype in which abnormal eosinophils are found (276). It is tempting to speculate that some of the consistent translocations may alter the expression of different genes whose normal function may be cell type or program specific, and therefore account for the various subtypes of leukemias. The cellular proto-oncogenes of possible relevance to AML in this regard might include c-mos which is located on the long arm of chromosome 8 that is involved in the t(8,21), or c-fes which is located on chromosome 15 (277).

Heterogeneity inherent in the patients' history is an important prognostic clue, in contrast to CML. AML may appear to have arisen "de novo" or may be preceded by a period of hematopoietic dysfunction often referred to as a preleukemic phase. In some instances, this appears to be related to (and result from) previous radiotherapy or chemotherapy given as treatment for another neoplasm (278). In other instances, contact with a chemical carcinogen appears to be the cause. However, because an acute marrow insufficiency accompanies the rapid accumulation of non-functional immature cells, events preceding this phenomenon may not be apparent and hence their nature missed. The term acute thus refers more to the rapid clinical progression of the disease and may have little to do with the initial pathogenetic mechanisms.

Despite the heterogeneity observed in different AML patients, which has led to the hypothesis that different diseases may be involved, treatment is similar and designed with the goal of obtaining a complete remission, i.e., a state where leukemic cells are undetectable by conventional morphological criteria. With current chemotherapy regimens, this can usually be achieved in 50-70% of cases (224), although results for AML evolving from preleukemia are less favorable (221).

AML: Cell of Origin. All AML cases studied to date have, like the myeloproliferative diseases, been shown to be clonal disorders (10). In AML, the neoplastic cells that appear unable to follow normal differentiation pathways usually predominate so that the cell of origin is difficult to ascertain. Evidence of some features of granulopoietic differentiation suggests that a precursor of at least this lineage is initially transformed. In some patients, it has been shown that both G6PD isoenzyme types are found in platelets, red blood cells and BFU-E, suggesting that the AML clone may have arisen in a granulocyte-restricted precursor, since other pathways were not affected (279). On the other hand, other cases have shown only a single isoenzyme type in the platelets, and erythroid cells, suggesting a commomn origin with the granulopoietic blasts (280). To account for this latter picture, it seems likely that in such patients, transformation occurs first in a pluripotent stem cell leading to clonal amplification, with later secondary changes resulting in failure of granulopoietic differentiation and the rapid accumulation of an abnormal blast population.

Differentiation in AML. AML has classically been considered to be a disorder in which cell differentiation is blocked at an immature stage. However, autoradiographic studies indicated that most of the blasts were non-dividing cells (281), and in this respect might be considered either as non-

cycling immature cells or as an abnormal type of end-cell. For a long time, the question was raised as to whether it might be possible to overcome this block, a concept that was viewed with skeptical optimism because of the therapeutic implications (282). It is now clear that under some circumstances, AML blast cells are able to acquire additional features of differentiated cells. Fresh leukemic cells can mature spontaneously in semi-solid assays, diffusion chambers or liquid cultures (283-288). The differentiating stimuli in these cultures have not been fully characterized but responsiveness of leukemic cells to factors present in leukocyte-conditioned media seems to be preserved (289,290). In addition, fresh AML cells or cell lines derived from AML cells have been shown to undergo various degrees of differentiation in response to a number of inducers, including tumor-promoting phorbol esters, DMSO and certain chemotherapeutic agents (291,292). Although the relevance of these latter observations to future therapeutic possibilities is totally unknown, evidence of a chemotherapy-induced clonal complete remission in AML has recently been reported (106,293).

Finally, the phenomenon of "lineage infidelity" characteristic of some AML clones deserves mention. This term refers to the finding in single cells of markers of more than one lineage (e.g., glycophorin and My-1) not normally expressed coincidentally. This phenomenon was first described in Friend leukemia (294) and K-562 (295) cells but has now been reported in a number of cases of AML by several different laboratories (296). Examples of co-expression of lymphoid and myeloid markers have been documented (297,298) as well as co-expression of markers of different myeloid lineages (296,299). In addition, it has been found that this lack of program regulation may be extrinsically modified by exposure of cells to 5-azacytidine (300). At the present time, the significance of these observations is unclear, although they

reinforce the idea that differentiation potential is not irreversibly blocked or fixed in all leukemic cells and that the genetic control of differentiation in leukemic cells may be less well regulated than in their normal counterparts.

Blast progenitors in AML. The production in vitro of small clusters and/or colonies of abnormal cells in assays of AML samples was first described in the 1970's (301,302). Improved conditions for blast colony growth have since been developed, the primary parameters being the use of a more potent source of stimulating factor (usually media conditioned by PHA-stimulated leukocytes) (303), a richer nutrient medium (304), and the removal of suppressive T cells that may co-exist in the cell suspension to be assayed (305). Evidence that the progenitors of blast colonies in vitro are a significant subpopulation of the leukemic clone responsible for blast cell production in vivo is based on (i) the demonstration of the same chromosome abnormalities (306,307) and G6PD isoenzyme type (10) in blast colonies as seen in direct marrow preparations, (ii) the finding of a significant correlation between blast progenitor numbers and the peripheral blast count (308), (iii) the finding that blast progenitors are actively cycling (in contrast to the blasts which are not) (309,310), (iv) the demonstration that blast progenitors sediment with a different profile than the total blast population (311), and (v) that the generation of new blast progenitors during blast colony formation in vitro correlates negatively with prognosis (312).

Although clinical findings indicate that normal hematopoietic stem cells persist in many patients with AML, it is clear that their granulopoietic potential is suppressed in vivo. When fresh AML marrow or blood samples are assayed in vitro, colonies of normally differentiated granulocytes and macrophages are also rarely observed (313). Information regarding other progenitor types is scanty (314). In general, these may appear to be decreased although this may

be more a function of their dilution by a large abnormal population than a true absolute decrease. Inhibition of normal differentiation by leukemic blast cells has also been described and attributed to the action of acidic isoferritins. However, these studies have been limited to the expression of CFU-C granulopoietic potential and therefore may not bear on the process of CFU-C production from pluripotent stem cells (315-319).

6. THESIS OBJECTIVE

The organization of the hematopoietic system into successive generations of committed precursors originating in a high proliferative pluripotent stem cell has been defined in man exclusively by short term clonogenic assays. As outlined above, these depend on the exogenous provision of growth-regulatory factors. Such systems are thus not well suited to the analysis of mechanisms regulating early hematopoietic cell behaviour (both normal and neoplastic) that are operative in vivo. Recent results obtained with the long-term culture system, as applied to murine bone marrow, indicate that stem cells can be maintained under these conditions for several months. Moreover, their proliferative activity can be readily manipulated, and a balanced pattern of self-renewal and production of differentiated progeny obtained without the exogenous provision of any known growth factors.

The first description of a human marrow culture system in which similar evidence of progenitor maintenance was obtained also showed a morphological organization of the culture that appeared similar to what is seen in the mouse, i.e., establishment of a complex adherent layer, above which granulopoietic cells could be found for periods of 2 months or more. These results suggest that it might be possible to use such a system to assess, and perhaps modulate, interactions between hematopoietic stem cells and stromal elements in human marrow populations.

The first objective of this work was: (i) to define the optimal conditions required for the maintenance of primitive hematopoietic cell types in long-term marrow cultures established from normal human marrow specimens, and to determine in a group of normal individuals the extent of interexperimental variation in progenitor cell recoveries and (ii) to establish if hematopoiesis was associated with non-hematopoietic cell types in the adherent layer and the categories of precursor cells that might be preferentially located there.

The second objective was to use this system to evaluate its potential for analyzing in vitro mechanisms that confer in vivo the property of clonal dominance to malignant precursor cells in CML and AML patients. The purpose was to investigate in both disorders whether the in vitro conditions generated in human long-term cultures would mimic the in vivo situation (i.e., reproduce the proliferative advantage of the neoplastic cells) or alternatively, whether they would allow expression in vitro of residual normal hematopoietic cells that are suppressed from differentiating in vivo. Chromosomal markers were used to differentiate between progeny of cytogenetically abnormal versus cytogenetically normal progenitor cells in all cases of CML and some cases of AML. In the AML studies, the kinetics of progenitors giving rise to abnormal (blast) clusters and colonies, and those giving rise to phenotypically normal colonies was also compared.

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C H A P T E R I I

ENZYMATIC TREATMENT OF LONG-TERM HUMAN MARROW CULTURES REVEALS THE PREFERENTIAL LOCATION OF PRIMITIVE HEMOPOIETIC PROGENITORS IN THE ADHERENT LAYER

1) INTRODUCTION

Long-term marrow cultures offer an in vitro approach to studies of stem cell proliferation and differentiation under conditions where close range interactions with stromal elements are likely to be operative (see Chapter I). With mouse marrow, it is now routinely possible to initiate long-term cultures with a single inoculum of cells and detect the release of significant numbers of stem cells and granulopoietic progenitors into the non-adherent fraction for periods of 2 months or more depending on the genotype (1-4). Convincing evidence of stem cell turnover has been obtained from thymidine suicide studies (5) and from the fact that the total number of stem cells removed as a result of each weekly medium change can eventually exceed the number in the original inoculum (6). This occurs even when all of the non-adherent cells are removed each week (2,3), suggesting that the adherent layer, in addition to providing conditions suitable for stem cell maintenance and proliferation, serves as the primary stem cell reservoir. Consistent with this is the finding that the self-renewal capacity of stem cells in the adherent layer at any given time is much higher than that displayed by their non-adherent derivatives (2).

At the time these studies were initiated, conditions that reproducibly support continuous granulopoiesis from a single inoculum of human marrow for periods of 1-3 months have just been described (7,8). Key parameters appeared

to be the use of a mixture of horse and fetal calf serum, with further supplementation of the growth medium with 10^{-6}M hydrocortisone and maintenance of the culture at 33°C. When these conditions were employed, assays of the non-adherent fraction showed that primitive granulopoietic progenitors were reproducibly detectable for an equivalent period, although the actual total numbers removed rarely exceeded input levels (7,8). In spite of this discrepancy between human and mouse cultures, certain similarities in their overall appearance were observed. Of note was the formation in the adherent layer of "cobblestone areas", which are thought to represent islands of primitive hemopoietic cells (7,8). In addition, in our initial studies, we noted the formation, just above the adherent layer, of a significant "pseudoadherent" cell layer. This layer is made up of large numbers of refractile cells that appear to be non-adherent, but are clearly not free-floating and fail to be removed when the growth medium is exchanged (Figure 3)(3). It therefore seemed likely that the adherent fraction of human cultures, like that of mouse cultures, might prove to contain a qualitatively and/or quantitatively more important population of progenitors than the non-adherent fraction. To test this hypothesis, a method for dissociating adherent layer cells was first developed. This method was then used to compare the numbers and types of the progenitors in both the non-adherent and adherent fractions of long-term human marrow cultures maintained for periods of up to 12 weeks.

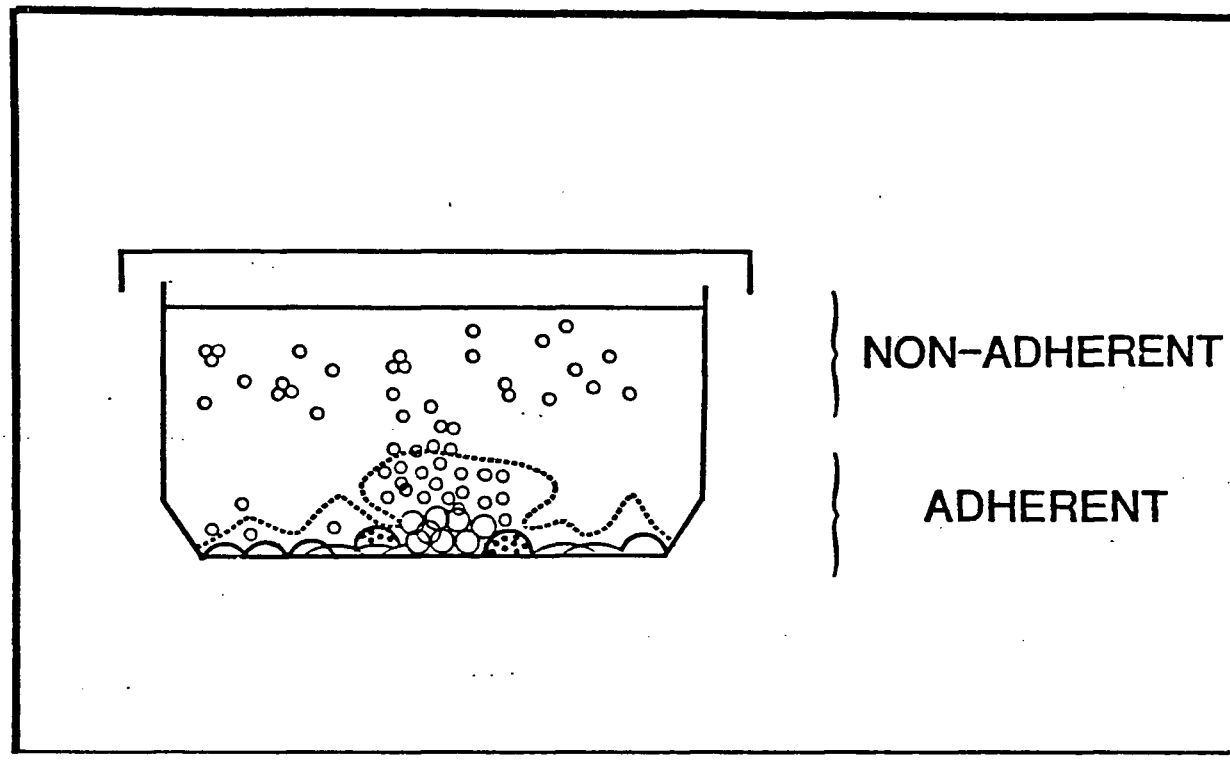


FIGURE 3. Schematic illustration of the 3-layer organization seen 2-3 weeks after the initiation of a long-term marrow culture

2) MATERIAL AND METHODS

(A) Patients

Marrow cells used were leftover samples from aspirates obtained with informed consent from 33 patients being investigated for neoplastic marrow involvment. Details of the diagnoses of these patients are given in Table I. The majority of the patients had non-Hodgkin's lymphoma or Hodgkin's disease. Most had received no prior chemotherapy or irradiation and none had been treated for several months prior to marrow aspiration. There were 19 males and 14 females. The average age was 50 years with a range from 9 to 85 years. All but 5 aspirates were subsequently found to be free of malignant involvment by morphological examination. The 5 involved marrows showed minimal infiltrates and progenitor levels in these were within our normal range (9). Five additional marrow specimens were obtained from normal individuals donating their marrow for transplantation (cultures # 63, 67, 78, 96, 108).

(B) Long-term Culture Methodology

Cells. All marrow specimens were collected in sterile tubes containing 800 units of preservative free heparin in 1 ml of α-medium. Only those containing $>2 \times 10^7$ cells per ml were used to initiate long-term cultures. The average marrow cell concentration in those used was $5.23 \pm 0.40 \times 10^7$ cells per ml for the 33 samples from patients and $5.34 \pm 2.19 \times 10^7$ cells per ml for the 5 samples from normal marrow transplant donors. Whatever the procedure chosen, long-term cultures were initiated by placing 2 to 3×10^7 nucleated cells in 8 ml of growth medium in a 60 mm x 15 mm tissue culture dish (Falcon). At the beginning of our studies, tissue culture dishes were selected rather than the usual 25 cm^2 flasks. This was because they were easier to handle in studies of the adherent layer, and seemed to yield more

TABLE I

Clinical Data for the 33 Patients Included in the Control Group

Culture No.	Age/Sex	Diagnosis	Marrow Infiltration
19	73/M	CARCINOMA (OAT CELL)	NO
*21	69/M	ANEMIA (UNEXPLAINED)	NO
23	66/F	CARCINOMA (OAT CELL)	NO
25	30/M	HODGKIN'S DISEASE	NO
27	75/F	MYELOMA	NO
*28	63/M	HODGKIN'S DISEASE	NO
35	58/M	MYELOMA	NO
*36	30/M	LYMPHOMA	NO
37	64/F	LYMPHOMA	YES
38	27/M	HODGKIN'S DISEASE	NO
*40	60/M	MALABSORPTION	NO
41	45/M	LYMPHOMA	NO
*42	31/F	LYMPHOMA	NO
45	80/F	CARCINOMA (BREAST)	NO
*46	85/M	CARCINOMA (PANCREAS)	NO
*55	55/M	HODGKIN'S DISEASE	NO
*56	45/M	HODGKIN'S DISEASE	NO
*57	33/M	HODGKIN'S DISEASE	NO
*58	33/M	HODGKIN'S DISEASE	NO
*60	41/F	LYMPHOMA	YES
61	32/F	LYMPHOMA	YES
*62	9/F	ALL REMISSION	NO
*66	39/F	HODGKIN'S DISEASE	NO
*68	67/F	OSTEOGENIC SARCOMA	NO
74	50/F	LYMPHOMA	NO
92	28/F	ANEMIA (CROHN DISEASE)	NO
*94	49/M	LYMPHOMA	YES
*97	71/M	ANEMIA (UNEXPLAINED)	NO
101	65/M	HODGKIN'S DISEASE	NO
*102	63/F	ANEMIA (IRON DEFICIENCY)	NO
*103	25/M	LYMPHOMA	NO
*107	57/M	HODGKIN'S DISEASE	NO
*109	32/F	LYMPHOMA	YES

* Cultures whose adherent layers have been processed.

nucleated cells and CFU-C in the non-adherent fraction. However, extensive comparisons were not made between the 2 containers.

Growth Medium. Two growth media were successively used. Both straight α-medium (10), or α-medium supplemented with extra glutamine (400 mg/l), inositol (40 mg/l), and folic acid (10 mg/l) when compared initially gave similar results (data not shown). The rationale for the use of the modified α-medium was to try a medium similar to the enriched Mc Coy's medium first used to maintain human long-term cultures (7). The original supplementation also included glucose (2 g/l), but this was rapidly withdrawn because it yielded a very acidic (yellow) medium that was assumed would be detrimental to the cultures if the feeding schedule was kept to once a week. Although we obtained no obvious evidence that the supplemented α-medium was superior by comparison to straight α-medium, it has been known for a long time that glutamine rather than glucose is the essential source of energy in many cultures (11), and visual inspection of our marrow cultures often showed those maintained in the supplemented α-medium to show a larger number of "cobblestone areas" in the adherent layer. Because of the lability of glutamine, supplemented α-medium was not kept for more than a week at $4^{\circ}C$.

The growth medium was supplemented with horse serum (HS, 12.5%, Flow laboratories), fetal calf serum (FCS, 12.5%, Flow laboratories), 2-β-mercaptoethanol ($10^{-4}M$), and hydrocortisone sodium succinate ($10^{-6}M$). Mercaptoethanol was made up to $10^{-4}M$ from a frozen stock solution of $10^{-2}M$, and hydrocortisone from a $10^{-4}M$ solution kept at $4^{\circ}C$ and made up fresh every week. Solutions of both of these reagents were made up in α-medium. Both horse and fetal calf sera were selected initially for their ability to support hematopoiesis in culture: the horse serum in mouse CFU-C assays, and the fetal calf serum in mouse and human CFU-E and BFU-E assays. Horse serum is usually

inhibitory in human CFU-E and BFU-E assays (12). The same batch of FCS was used throughout this study. Three batches of horse sera were used. Later batches of horse sera were chosen because they gave equivalent progenitor maintenance in long-term human marrow cultures, although some variation in the extent of fat cell proliferation was noted between batches.

Initiation of Long-term Cultures. Two methods were compared. These are referred to as method A and method B. In method A, dishes were inoculated with cells from unprocessed bone marrow aspirates and no effort was made to obtain a single cell suspension. This was based on the notion that disruption of the granules has been thought to be detrimental to the establishment of cultures and their subsequent ability to yield progenitors (7,13). Dishes (60 mm x 15 mm) were placed in 100 mm x 20 mm dishes (Falcon), to decrease the probability of contamination. After an initial 3-4 days of incubation, all the non-adherent cells were removed and layered over Ficoll-Hypaque (density 1.077 g/cm^3, LSM, Litton-Bionetics) or Percoll (density 1.075-1.077 g/cm^3, Pharmacia, Sweden). Red cells and mature granulocytes were discarded and the light density cells then washed twice in α-medium with 2% FCS, and returned to their original dishes in fresh medium. This step appeared to be necessary to avoid the accumulation of lysed red blood cells which appeared to enhance the development of multinucleated phagocytic macrophages, a cell type I associated otherwise with old, declining cultures. This separation step was not undertaken when cultures were initiated with highly cellular marrow specimens (initial concentrations above 7 x 10^7 cells/ml) where red blood cell contamination is minimal. Initiation of long-term marrow cultures with Ficoll-Hypaque separated marrow cells was not evaluated since in the mouse system, this has been shown to give less than optimal results (14). In addition, such an initial separation procedure would also remove plasma which

may contribute components, such as fibrinogen for example, which can enhance progenitor yields from such cultures (15).

In method B, cultures were initiated with unwashed buffy-coat cells suspended in the marrow plasma and growth medium at the same concentration (2-3 x 10^7 cells per dish) as used for method A. The buffy coat preparation was obtained by a light centrifugation (200 g, 4 min), sometimes followed by a 20-30 minute gravity sedimentation to remove contaminating red cells more completely. The advantage of this method was that it eliminated the need to remove red cells 3-4 days later by the separation of the non-adherent cells on Ficoll-Hypaque. As a result, this step at day 3-4 was simply reduced to a total change of the growth medium.

In both methods, dishes were incubated during the first 3-4 days at either 33°C or 37°C and then transferred to 33°C, in all cases in a humidified atmosphere of 5% CO_2 in air. The superiority of the 33°C incubation temperature for stem cell maintenance was first reported by Dexter et al (1). Adoption of an initial incubation at 37°C was based on the observation that this appeared to promote a more rapid establishment of the adherent layer. Maintenance of cultures constantly at 37°C resulted in frequent early detachment of the adherent layer.

Maintenance. At weekly intervals, the growth medium was either totally replaced (method A) or only half-replaced (method B). In both cases, half of the non-adherent cells were removed. These were used for nucleated cell counts, and for assessment of the progenitor content by cloning aliquots in standard methylcellulose cultures. The number of colony-forming progenitors present in the aliquot of marrow used to initiate each long-term marrow culture was calculated from the number of colony-forming cells recovered in the light-density fraction of a known volume of starting marrow aspirate after

separation on Ficoll-Hypaque or in an aliquot of the buffy-coat preparation actually used to initiate the cultures.

All the feeding procedures were done very gently. New growth medium was added by letting it flow slowly down the side wall of the dish to avoid disturbing the adherent layer or even the viscous pseudo-adherent layer. Examination of the cultures at this step showed that many of the cells in the pseudo-adherent layer were left behind after the medium has been removed, which meant that calculations of the numbers of cells in the non-adherent fraction excluded this intermediate component.

Enzymatic Detachment of the Adherent Layer. Bacterial collagenase type I (200 units/mg protein, Sigma chemicals) was dissolved in calcium and magnesium-free Hanks balanced salt solution (HBSS-Ca-Mg) to a final concentration of 0.13% and the solution sterilized by passage through a 0.22u millipore filter. This solution was kept at 4°C and used within 48 hours. Just before use, FCS was added to give a final concentration of 20% FCS and 0.10% collagenase. To detach adherent cells, non-adherent cells and all the growth medium were first removed from the culture dish. Cultures were then vigorously washed twice with fresh HBSS-Ca-Mg, and the additional detached cells then added to the non-adherent suspension. Ten ml of the 20% FCS in 0.10% collagenase solution were then pipetted onto the adherent layer and the cultures incubated undisturbed for 3 hours at 37°C in an atmosphere of 5% CO_2 in air. At the end of the incubation period, many cells were completely detached (Figure 4) and most of the remaining adherent cells could be readily resuspended by gentle but sustained pipetting.

Adherent cells were centrifuged at 180g for 10 min at room temperature, and then washed twice in 2% FCS in HBSS-Ca-Mg. After each centrifugation, the cell pellet was carefully resuspended to minimize the formation of clumps.

FIGURE 4. Appearance of the adherent layer of a long-term marrow culture after incubation with collagenase (x40)

Using this procedure, it was routinely possible to achieve a suspension where most of the cells were present as single cells. When occasionally clumps of cells were observed, they were allowed to settle out by a short gravity sedimentation. Assessment of cell viability by dye exclusion (1% Nigrosin), routinely showed less than 20% of the cells stained. Visual assessment of long-term cultures after treatment with collagenase showed that some fibroblast-like cells usually remained on the bottom of the dishes. These could be subsequently detached by trypsinisation (as described below), and were shown to constitute 26 $\pm$ 3% of the total adherent layer (average of 5 experiments). Preliminary experiments established that there were no progenitors in this collagenase-resistant adherent cell population. Therefore, the population released by collagenase was slightly enriched for progenitors by comparison to the complete adherent cell fraction released by trypsin.

Bacto trypsin (Difco laboratories, Detroit, Michigan), was dissolved in citrated saline (pH 7.0) to a concentration equivalent to 0.1% of trypsin Difco 1/250. Cultures were prepared as for the collagenase procedure. However, no FCS was added to the trypsin solution and cultures incubated 10 min at 37^{o}C in 5 ml of the enzyme solution. One ml of FCS was added then to stop further trypsin action, and all adherent cells readily detached with light pipetting.

In order to evaluate whether the enzymatic treatment by itself had any influence on the colony-forming ability of the various types of hematopoietic progenitors being assayed, the following experiment was performed. Fresh normal marrow cells were separated on Ficoll-Hypaque, and 2 x 10^{6} cells from the light density fraction incubated at 37^{o}C in petri dishes (not tissue culture dishes), either in 5 ml of trypsin solution for 10 min, or in 10 ml of

collagenase solution (+20% FCS) for 3 hours, or in 10 ml of the same medium without collagenase. At the end of the incubation period, the cells were washed twice in HBSS-Ca-Mg containing 2% FCS, and assayed in methylcellulose. Cell recovery after incubation was always greater than 90%. Each different lot of collagenase and trypsin used in the study was tested one or two times in this way.

Two problems were constantly present in the handling of long-term cultures. Contamination was the major one. Bacterial contamination was avoided by supplementation of the growth medium with 100 units/ml of penicillin, and 100 ug/ml of streptomycin. Fungal and yeast contamination was effectively minimized by care in the use of fresh medium, avoidance of spillage and the separate handling of each culture dish. The second problem was detachment of the adherent layer, which was usually sudden and irreversible, and since all of the cells ended up in a tight ball, signified the loss of the culture. This rarely occured before the third week, when confluence was reached, and appeared to be related to the thickness of the adherent layer.

(C) Hemopoietic Colony Assays

Erythropoietic (CFU-E and BFU-E), granulopoietic (CFU-C), and pluripotent (CFU-G/E) progenitors were assayed in 0.8% methylcellulose in α-medium or Iscove's medium, supplemented with 30% FCS, 1% deionised BSA buffered with bicarbonate (1 ml of 7% bicarbonate for 40 ml of 10% BSA), 10^{-4}M 2-β-mercaptoethanol and 200 mM L-Glutamine. Two and a half to 5 units per ml of human erythropoietin (Ep, purified in the Terry Fox Laboratory laboratory to a specific activity of at least 100 units per mg of protein (16)), was added and 4.5% of phytohemagglutinin (PHA)-stimulated human leukocyte conditioned medium

used as a source of BPA and CSA. PHA-conditioned medium was prepared by incubating blood buffy coat leukocytes (separated in 0.1% methylcellulose) at a concentration of 1 x 10^6 cells per ml with 0.5% PHA (Gibco, M-type), in Iscove's or α-medium with 10% FCS. Incubation was performed at 37°C during 7 days, and at the end of the incubation period, the medium was collected, spun at 2000 rpm to remove cells and cell debris, and then stored in frozen aliquots. Erythropoietin and conditioned medium preparations were both calibrated against standard preparations to ensure equivalence of activity in different batches.

Cells were plated in methylcellulose assays at a final concentration of 0.5 to 2 x 10^5 cells per 1.1 ml of culture. Buffy coat cells of the starting assays were plated at 2 x 10^5 cells per 1.1 ml; light density marrow cells of the starting assays at 1 x 10^5 per 1.1 ml. Non-adherent cells of long-term cultures were usually plated at 0.5 x 10^5 cells per 1.1 ml during the first 2-3 weeks, because of the increased concentration of CFU-C in the non-adherent fraction during that period, and then subsequently at 1 x 10^5 cells per 1.1 ml. After 6-8 weeks, the number of cells recovered in the non-adherent fraction was often reduced so that as few as 1 to 2 x 10^4 cells was all that could be plated. Adherent cells were plated at 1 x 10^5 or 0.5 x 10^5 cells per 1.1 ml. As the adherent cell suspension was a mixture of some hemopoietic cells and many non-hemopoietic cells (including fibroblasts), it was found that it was important to select assay petri dishes that strongly prevented any spreading of the adherent cells during the 3 week period required for hemopoietic colony growth. If this was not done, fibroblast proliferation was extensive and inhibition of erythroid, and to a lesser extent of granulocytic, colony growth resulted. Proliferation of fibroblasts in the methylcellulose assays also led to attachment of cells from colonies into the adherent sheet that formed and this made colony assessment difficult.

Each assay was set up in duplicate or quadriplicate and scored 2 or 3 times to obtain reliable counts of both small and large erythroid colonies using the following criteria: isolated single or paired clusters of CFU-E derived erythroid cells were counted at day 7 or 8 after plating. Bursts containing 3-8 clusters were scored at day 12-14, and bursts containing more than 8 clusters after 16-18 days. In the latter category, bursts containing 9-16 and more than 16 clusters were recorded separately (17). However, in most instances, BFU-E numbers have been added together and presented as a single value.

Colonies of granulocytes and macrophages (from CFU-C) containing more than 20 cells were scored in the same assay dishes as used for CFU-E and BFU-E estimate. Scoring was done on day 16 of incubation to minimize confusion with immature erythroid colonies since mature erythroid and granulocyte macrophage colonies are readily distinguished by their different characteristic morphologies.

Mixed colonies (from CFU-G/E) were identified by the presence of both granulocytic and erythroid elements in single isolated colonies. When the number of BFU-E and CFU-C plated was sufficiently low to allow meaningful assessment of mixed colonies, these were scored separately after 16-18 days of incubation. The accuracy of colony identification by direct visualization was confirmed by occasional staining of cytospun colonies with May-Grunwald-Giemsa.

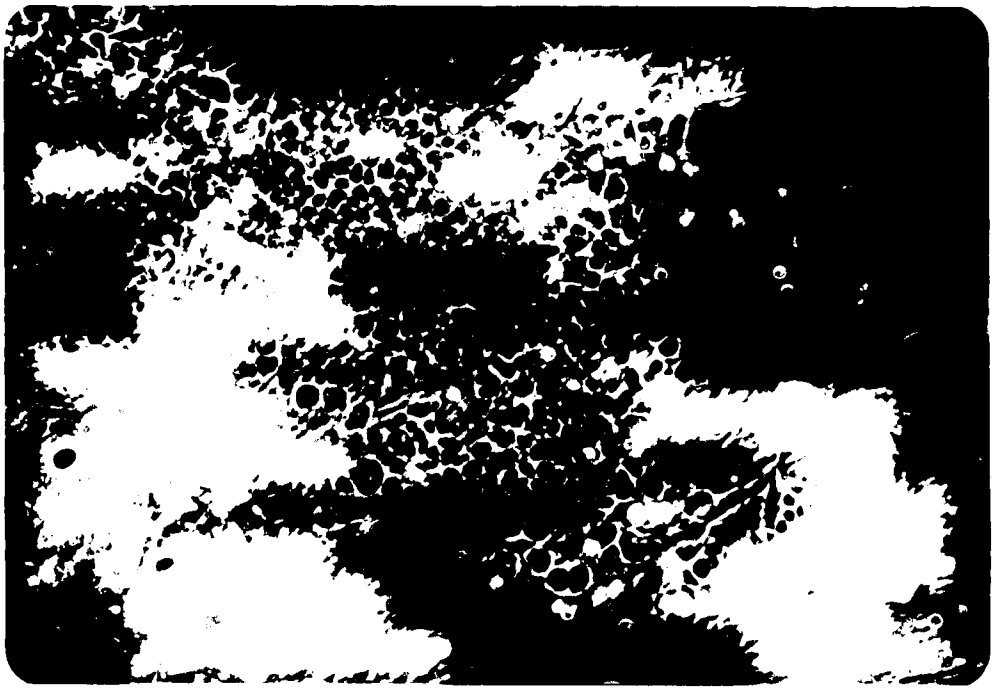
3) RESULTS

(A) Gross Morphology of the Cultures as a Function of Time

Periodic inspection of cultures under an inverted microscope showed that the adherent layer formed primarily by the outgrowth of cells from marrow granules also rich in fat cells and erythroblasts that attached to the bottom of the culture dish during the first hours. In our experience, the presence of these aggregates was predictive of the rapidity of development of the adherent layer and this in turn was related to the successful maintenance of hematopoiesis. Such granules were especially numerous in "reactive" marrows, such as lymphomas and anemias. From these aggregates, elongated fibroblast-like cells radiated out and between their cytoplasmic projections clusters of large round adherent cells could be seen in close association with them (Figure 5A). The adherent layer became confluent around week 3. Later, large cobblestone areas of flattened confluent cells, reminiscent of those initially seen primarily adjacent to the granules, appeared embedded firmly in the heavy network of other adherent elements (Figure 5B). These could not be readily released by simple pipetting. The presence of hematopoietic cells within the adherent layer was clearly shown on May-Grunwald-Giemsa stained slides (Figure 6). These included some early and mature granulopoietic cells, rare plasma cells, and occasional lymphocytes (4-5% of the total adherent fraction) which were found to be T-lymphocytes by rosetting with sheep red blood cells (18). The non-hematopoietic components of the adherent layer were not further characterized. After 8-10 weeks, there was usually a progressive accumulation of brownish, fat-laden macrophages which organized in lightly adherent clusters (Figure 5C). This picture was often subsequently found to be associated with a decline in hematopoiesis.

(A)

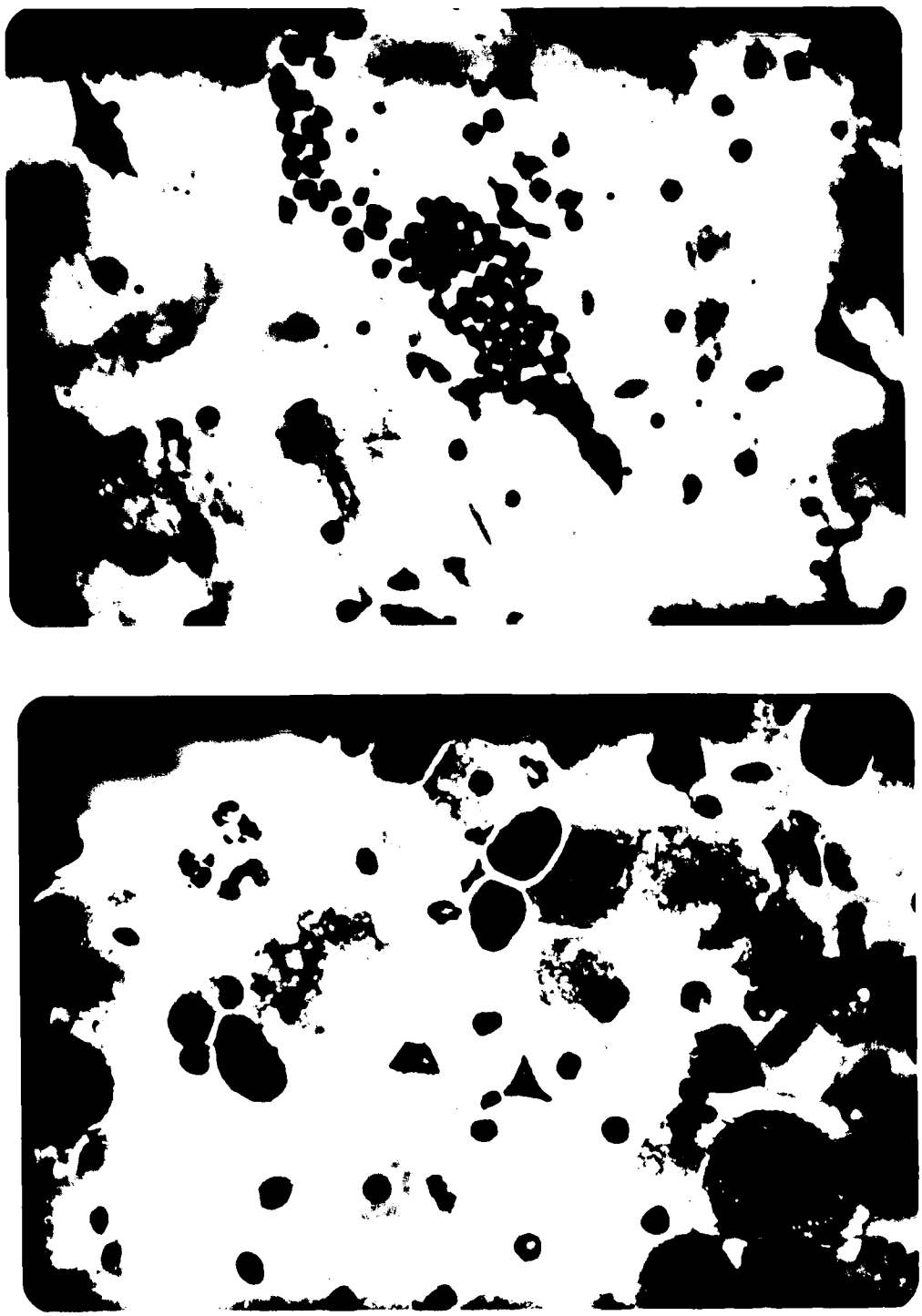
FIGURE 5. Morphological appearance of the adherent layer of long-term marrow cultures at different times after initiation.
(A) 4 days - an initial adherent island (x40)



(B)

(C)

FIGURE 5. (B) 4 weeks - a cobblestone area of putative hemopoietic cells (x200)
(C) 15 weeks - accumulation of fat-laden macrophages and disruption of the adherent layer (x80)



(A)

(B)

FIGURE 6. Morphological appearance of the adherent layer of a long-term marrow culture as shown by May-Grunwald-Giemsa staining
(A) In situ staining of a cobblestone area (x160)
(B) Staining of cytospun adherent cells detached by collagenase (x400)

B) Variation of Nucleated Cell and Progenitor Cell Content of the Non-Adherent Fraction with Different Initiation and Feeding Procedures

Thirty-eight cultures were initiated with unprocessed marrow. Thirty-two of these were fed by total medium change (method A) and six were fed by half-medium change (method B). Eight cultures were initiated with buffy coat cells (method B). Three of these were fed by total medium change (method A) and five by half-medium change (method B). As shown in Figure 7, initiation with buffy coat appeared to be at least as good as initiation with unprocessed marrow, although a significant improvement, as suggested in Figure 7, was not consistently obvious. This was not the case when the two feeding procedures were compared. As shown in Figure 8, the half medium change gave a higher yield of progenitors in the non-adherent fraction that was particularly marked after 4 weeks, although there was no change in the total cellularity of the non-adherent fraction.

The general evolution of hematopoiesis was the same in all cultures and showed the following pattern (Figure 8). Initially, there was a rapid decline in the total numbers of non-adherent cells, and a parallel decrease in nucleated as well as progenitor cell content which appeared independent of the culture conditions. By 2-3 weeks, the total number of non-adherent cells had thus on average dropped to 3-5 % of the number of nucleated cells present in the initial inoculum. At the same time, the number of non-adherent BFU-E (80-200) had reached approximately 1% of the input value, and the number of non-adherent CFU-C (500-2000) had reached approximately 10% of the input value. Between weeks 4 to 8, the decline in both total non-adherent cell numbers and in non-adherent progenitor cell numbers was slower, frequently with oscillations from one week to another when individual experiments were followed.

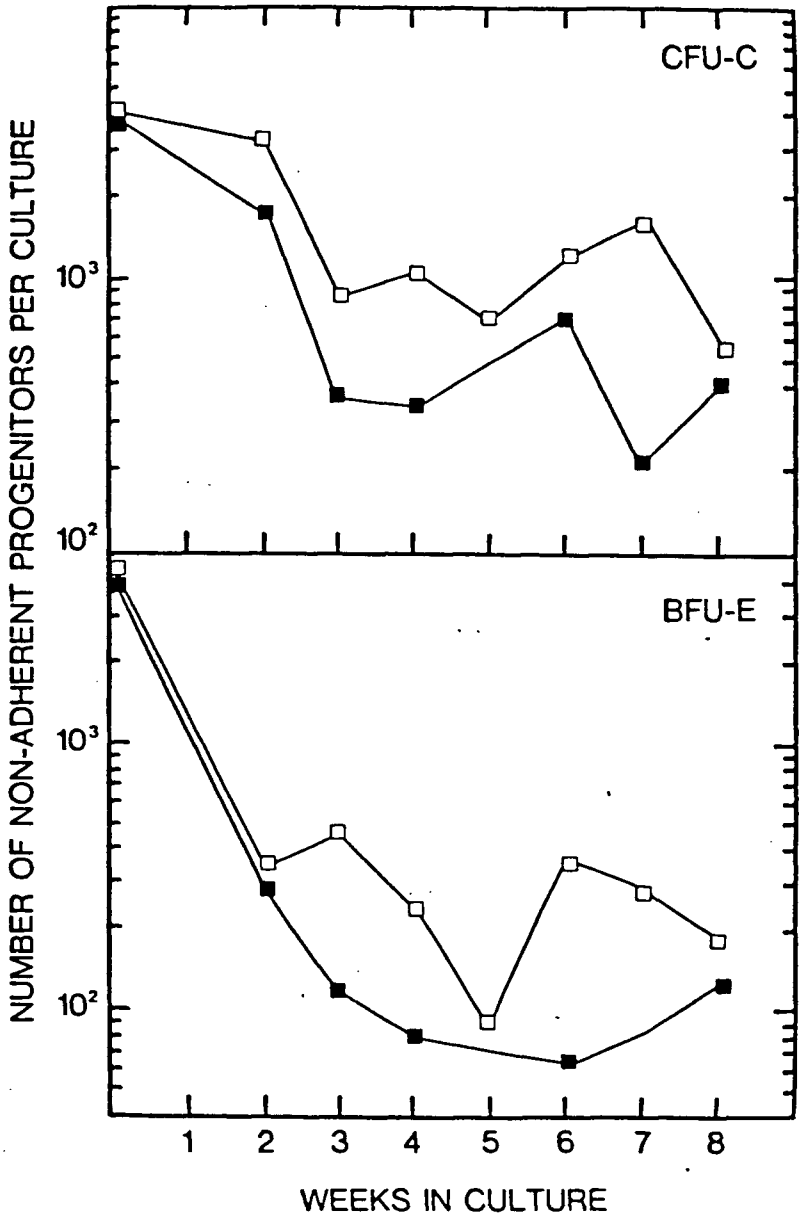


FIGURE 7. Comparison of the effect of two initiation procedures on the progenitor content of the non-adherent fraction: Initiation with either buffy-coat marrow cells (□) or unprocessed marrow cells (■) from the same specimen

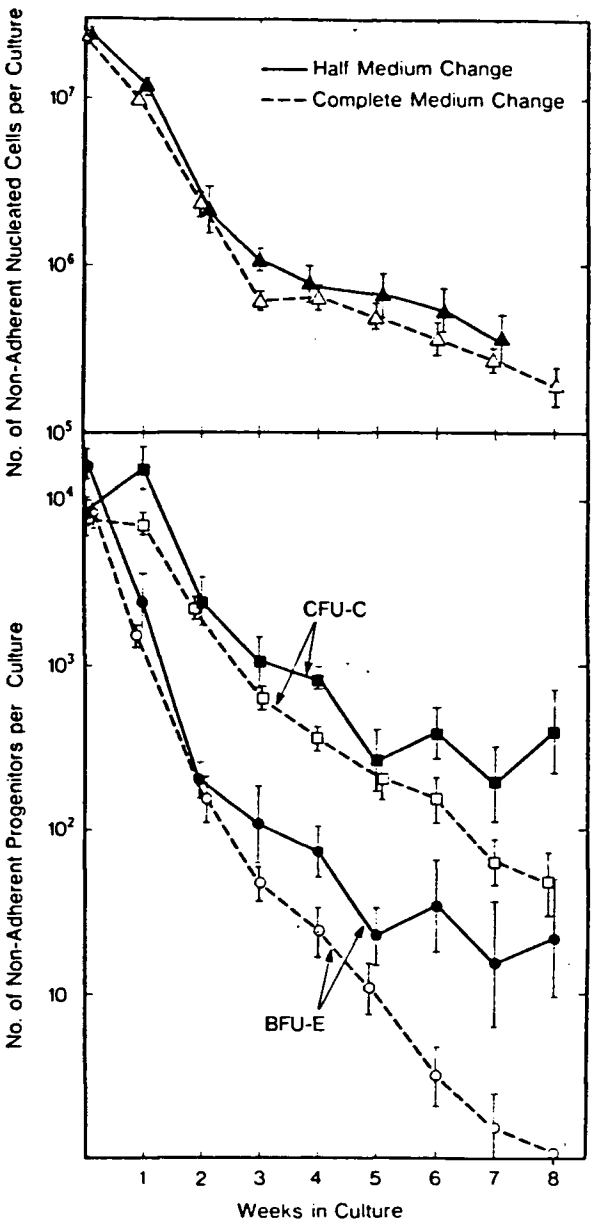


FIGURE 8. Comparison of the effect of two different feeding procedures on the total number of nucleated cells (upper panel) and progenitors (lower panel) detected each week in normal long-term cultures. Half-medium change data are from 13 different experiments, complete medium change data from 34 different experiments. Each point represents the geometric mean ± SEM of results obtained from different experiments.

The decline in the progenitor content of the non-adherent fraction with time was considerably slower when only half of the medium was exchanged. Under these conditions, BFU-E were detected in the non-adherent fraction on average for 7-8 weeks (i.e., in 6 of the 7 cultures carried for that length of time). CFU-C were detected in the non-adherent fraction of all cultures for 8 weeks. However, the total number of non-adherent progenitors was small after 6 weeks (1-100 BFU-E and 50-600 CFU-C at week 8), and considerable interexperimental variation seen. This was not explained by intraexperimental variation that did not exceed 20%. The rapid initial decline in non-adherent BFU-E and CFU-C numbers usually resulted in a cumulative total number removed that failed to surpass input values. Evidence for an expansion of the BFU-E population on this basis was thus never obtained, and in the case of CFU-C was at best limited to increases occurring in the first two weeks (Figure 9).

CFU-E were rarely detected after 2-3 weeks, in spite of the presence of BFU-E. An analogous block in erythropoietic cell differentiation is typical of the murine long-term marrow culture system also (1). Progenitors of mixed granulocyte/erythroid colonies (from CFU-G/E) were frequently detectable in the non-adherent fraction for periods of 4 weeks (Table II), although accurate assessment of their numbers in one and two week assays was usually hampered by the increased concentrations of CFU-C at these times.

Cultures were usually not carried beyond 8 weeks, but were sacrified at or before this time to allow assessment of the progenitor content of the adherent layer (see below).

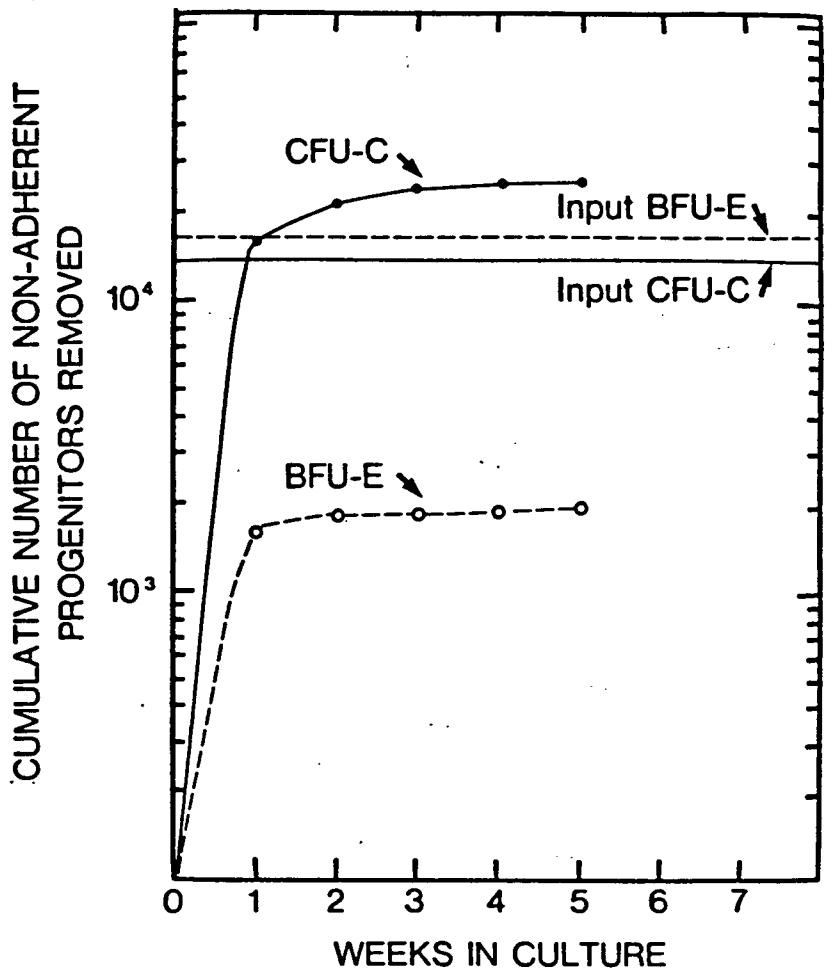


FIGURE 9. Cumulative number of progenitors removed each week from the non-adherent fraction of a long-term culture initiated with normal marrow

TABLE II

Comparison of CFU-G/E Recoveries in the Adherent Layer (A) Versus the Corresponding Non-Adherent Fraction (NA) of Long-Term Cultures Terminated after Varying Periods of Incubation*

Age of Culture (wks)	CFU-G/E Per Culture A : NA
2	42 : 13
	120 : <32†
	120 : 40
	99 : <33†
	160 : 80
3	39 : <6†
	<19† : 9
	<7† : 12
4	40 : 7
	<25†^{O} : 18^{O}
	60^{O} : <36†^{O}
5	22 : <1†
6	25 : <25†
7	8 : <2 †

* Conservative estimates based on the mean of counts from 2 dishes. Each value corresponds to a different experiment.

^{O} Two cultures initiated with the same marrow and assayed separately.

† Maximum value if 1 colony had been seen in any of the assay dishes scored.

(C) Comparison of the Cellularity and Composition of the Adherent versus the Non-adherent Fractions

Lack of Toxicity of either Collagenase or Trypsin. Normal marrow cells were treated with collagenase or trypsin according to a protocol designed to mimic as much as possible the method used to harvest the cells from the adherent layer (for details, see Materials and Methods). The results of 2 experiments are shown in Table III. Detection of erythropoietic and granulopoietic progenitors in fresh marrow was not significantly affected by either enzymatic treatment, although the number of very large erythroid colonies (more than 16 clusters) was usually slightly decreased in the treated group (data not shown). Otherwise, no morphological differences was noted between the colonies obtained from test or control cell suspensions. Also, the low Ca and Mg content of the incubation medium, provided by supplementing HBSS-Ca-Mg with 20% FCS did not appear to have any adverse effect on plating efficiency for periods up to 3 hours at 37°C. The low Mg and Ca conditions were used to minimize intercellular adhesion and yet allow effective collagenase activity.

Nucleated Cells and Progenitor Cell Recoveries from the Adherent Layer. Thirty-one cultures initiated with marrow from 16 different marrow specimens according to method A, and fed according to method A, were sacrified for parallel assessment of their non-adherent and adherent populations. In 6 instances, several cultures were set up with the same marrow and harvested at different times, and in 6 cases, results were also obtained from replicate cultures harvested at the same time but assessed separately. In contrast to the oscillations seen in the total non-adherent cell counts for the first 8 weeks, the cellularity of the adherent fraction in a given experiment was found to remain relatively constant between 2 and 8 weeks, and little inter-

TABLE III

Effects of Collagenase and Trypsin Treatment Procedures on the Plating Efficiency of Hemopoietic Progenitors

Expt No.	Incubation Conditions	CFU-E*	BFU-E*	CFU-C*
1	No incubation	124 ± 12	120 ± 14	374 ± 28
	3 hrs at 37°C-medium	148 ± 22	134 ± 6	396 ± 40
	3 hrs at 37°C-collagenase, lot #1	226 ± 20	192 ± 20	452 ± 20
	3 hrs at 37°C-collagenase, lot #2	210 ± 10	150 ± 22	516 ± 10
2	No incubation	142 ± 16	62 ± 12	56 ± 9
	3 hrs at 37°C-medium	204 ± 10	79 ± 14	116 ± 16
	3 hrs at 37°C-collagenase, lot #2	201 ± 26	76 ± 5	110 ± 6
	3 hrs at 37°C-collagenase, lot #3	210 ± 11	96 ± 7	101 ± 5
	10' at 37°C-trypsin, lot #1	193 ± 19	76 ± 7	70 ± 6

* Values shown are the number of progenitors detectable per 10^5 marrow cells (mean ± 1 SEM of counts from 3-4 dishes).

experimental variation was encountered. The average total cellularity of the adherent layer during this interval was $2.8 \pm 0.3 \times 10^6$ cells.

The results of methylcellulose assays from this group of experiments are shown on Figure 10. BFU-E (Figure 10A) were detected in the adherent layer of every culture although these were not always present in detectable numbers in the non-adherent fraction after 4 weeks. Except for 3 early cultures, (ended at 2 or 3 weeks), more BFU-E were always present in the adherent layer by comparison to the non-adherent fraction of the same culture. Thus, although the ratio of adherent BFU-E to non-adherent BFU-E numbers between 2 and 3 weeks was on average only 2-3, this ratio increased to a value of approximately 10 by week 6. The concentration of BFU-E in the adherent layer also was higher than the concentration of BFU-E in the non-adherent fraction, and this relative enrichment of BFU-E in the adherent layer increased with time. If the concentration were expressed in terms of BFU-E per number of hematopoietic adherent cells, it would appear even higher. However, as these cells were not easily enumerated, this was difficult to document. Most of the BFU-E detected after 4 weeks generated large erythroid colonies containing more than 8 clusters. Erythroid colonies produced by BFU-E from younger cultures included a significant component of smaller colonies. CFU-E, which rapidly declined to low levels in the non-adherent fraction were also rarely detected in the adherent layer. CFU-C were detectable in both fractions in every experiment, but as shown in Figure 10B, their number decreased progressively with time in culture. This occured in both fractions at more or less similar rates. However, after 4 weeks, the CFU-C content of the adherent layer was on average slightly higher (1.5-2 fold) and the disparity in total cellularity of the 2 fractions (described above) resulted in the concentration of CFU-C in the non-adherent fraction remaining consistently higher.

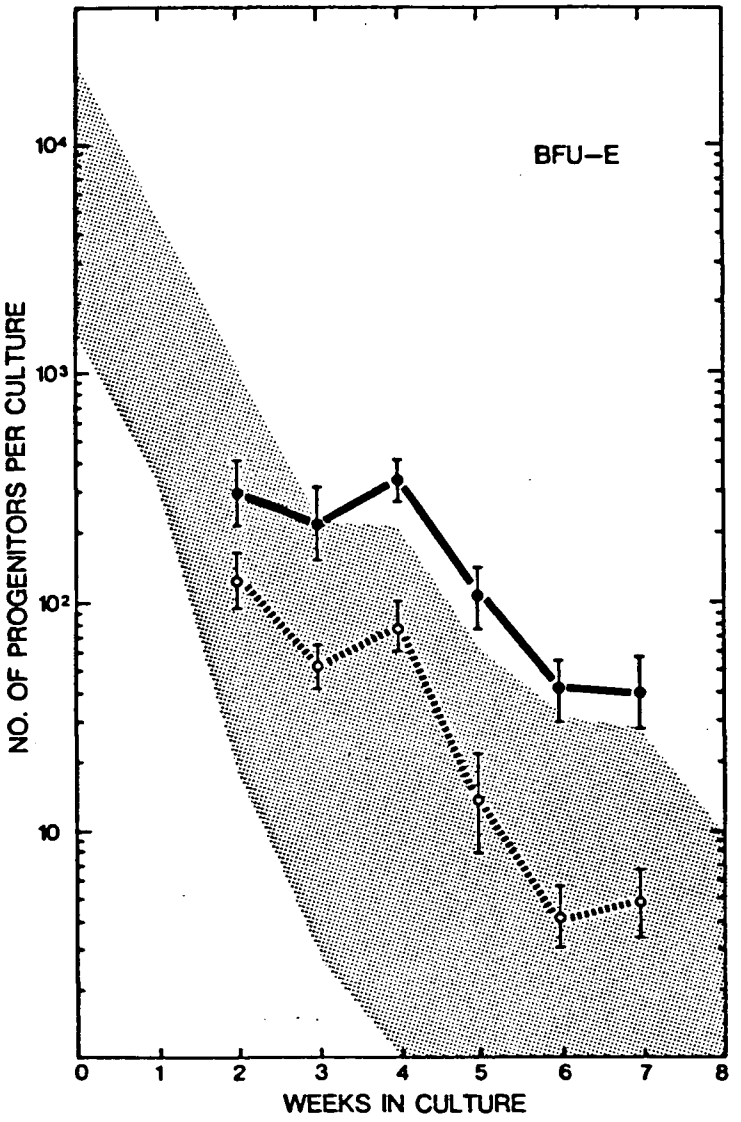


FIGURE 10A. BFU-E content of human long-term marrow cultures (initiated and fed by method A) after varying periods of incubation. Solid line: Recovery of BFU-E in the adherent layer; dashed line: Recovery of BFU-E in the non-adherent fraction; shaded area: Range of non-adherent BFU-E values for 22 normal long-term marrow cultures. Each point represents the geometric mean ± SEM of results obtained in 4-6 different experiments. The week 7 point represents the pooled results of experiments ended at week 7 and 8

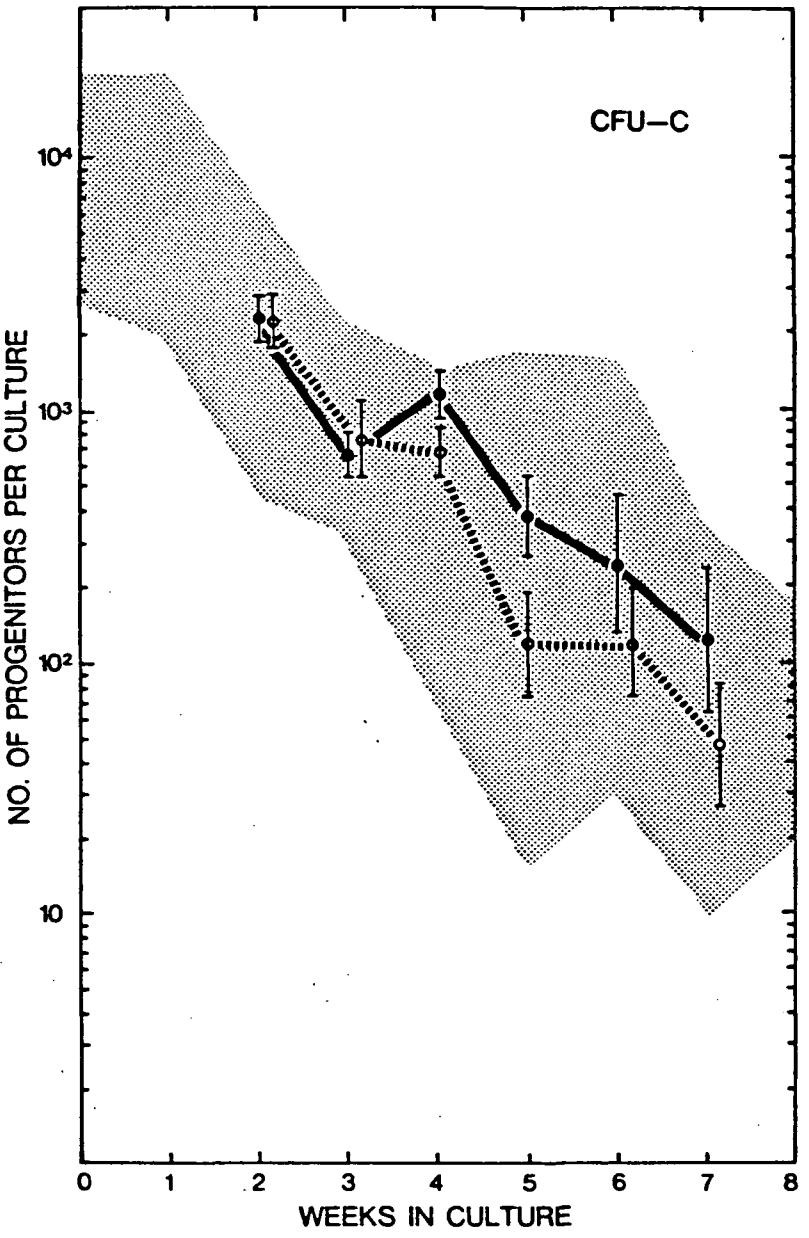


FIGURE 10B. CFU-C content of human long-term marrow cultures (initiated and fed by method A) after varying periods of incubation. Solid line: Recovery of CFU-C from the adherent layer; dashed line: Recovery of CFU-C from the non-adherent fraction; shaded area: Range of non-adherent CFU-C values for 22 normal long-term marrow cultures. Each point represents the geometric mean ± SEM of results obtained in 4-6 different experiments. The week 7 point represents the pooled results of experiments ended at week 7 and 8

Although similar numbers of granulocyte-macrophage colonies were obtained from non-adherent and adherent fractions, the morphology of the colonies produced was markedly different. Colonies derived from non-adherent CFU-C rarely achieved a size of more than 500 cells and usually included a major component of small macrophage colonies. Most of the CFU-C in the adherent layer generated colonies that were predominantly granulocytic, and between 5 and 20% yielded very large, compact granulocytic colonies (containing 10^3-10^5 cells). Colonies of this type (Figure 11) were never observed in assays of the non-adherent fraction after 2 weeks.

Mixed G/E colonies could be reliably detected in assays from 14 cultures (12 experiments). In 11 of these, CFU-G/E were found to be preferentially located in the adherent layer (Table II). The unequal distribution of CFU-G/E between both fractions and their decline during the culture period followed the same pattern as that described above for BFU-E.

As shown in Table IV, the other methods for initiating and maintaining long-term marrow cultures gave consistently the same or improved progenitor yields in both fractions. Thus, the majority of all progenitor types still remained in the adherent layer. This meant that the overall maintenance of hematopoiesis has been improved and that the inequal distribution of progenitor cells between the adherent and non-adherent fractions (Figure 12) was likely to be a feature of functional long-term marrow cultures independent of the initiation or maintenance procedure used.

FIGURE 11. Photomicrograph of a typical large granulocyte colony observed in a 17 day old methylcellulose assay of a long-term marrow culture adherent layer (X 100)

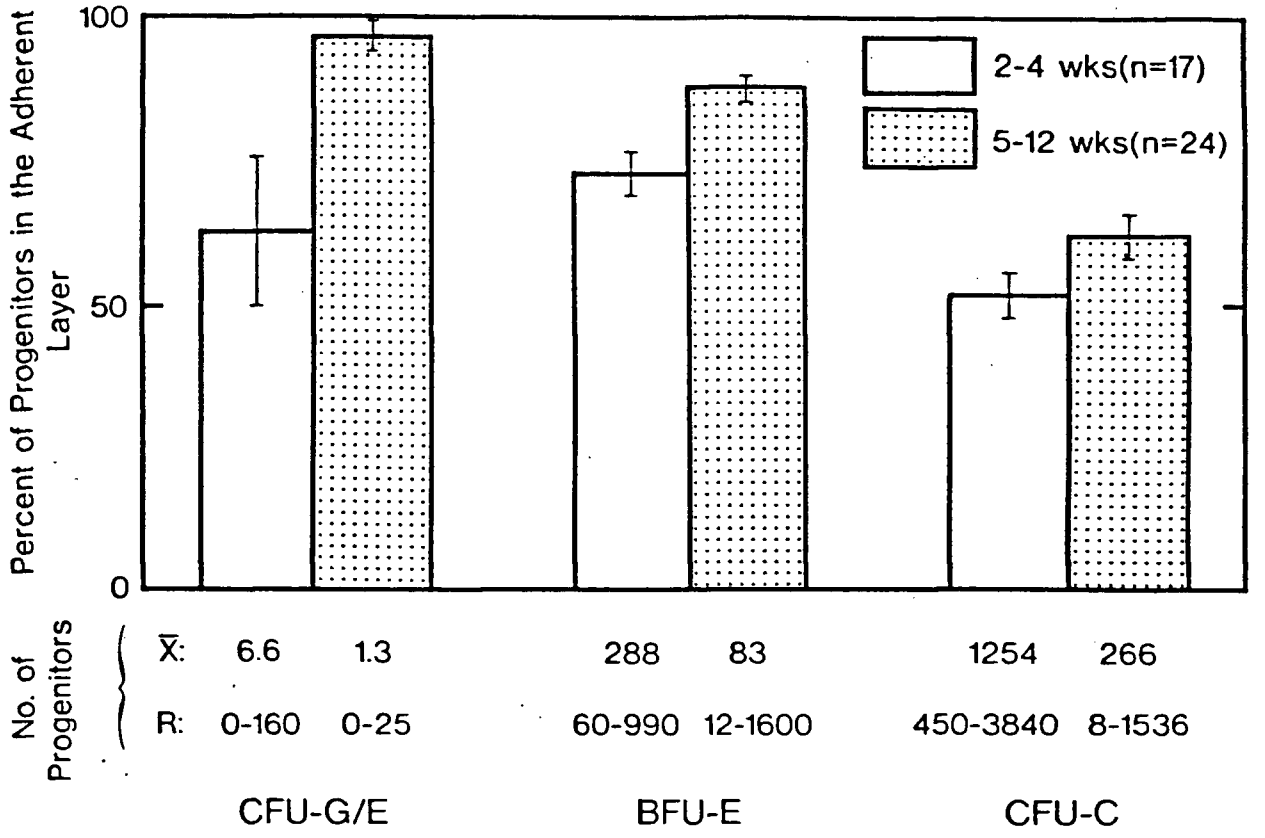


FIGURE 12. Pooled results from 41 experiments where paired data for the progenitor content of the non-adherent and adherent fractions of individual normal human long-term marrow cultures were obtained

TABLE IV

Progenitor Cell Content of the Adherent and the Non-Adherent Fractions of Long-Term Cultures Initiated and Fed with Different Procedures

Culture No.	Initiation/Feeding		Age of Culture (wks)	BFU-E A:NA	CFU-C A:NA	CFU-G/E A:NA
94	B	A	12	48 : 1	105 : 50	<16* : <2*
97	A	A	8	144 : <3*	40 : 10	<11* : <3*
	A	B	8	198 : 27	134 : 56	7 : <3*
102	A	A	6	2,025 : <5*	1,350 : 23	15 : <5*
	B	B	6	1,600 : 163	1,130 : 343	21 : <2*
	B	A	9	329 : 8	714 : 199	<24* : <4*
	B	B	9	990 : 45	1,106 : 684	<25* : <5*
103	A	B	8	7 : <3*	58 : 97	<14* : <3*
	B	B	8	59 : <2*	117 : 54	<12* : <2*
107	B	B	6	252 : 171	266 : 983	10 : <3*
108	B	B	5	336 : 108	1,536 : 3,689	5 : <15*
	B	B	5	50 : 5	837 : 790	20 : <5*
109	B	B	5	110 : 56	235 : 959	10 : 3.5

* No progenitors of this type detected. Value shown is that corresponding to 1 colony detected in any of the assay dishes scored.

4) DISCUSSION

In this study, we have shown that primitive erythropoietic, granulopoietic and pluripotent progenitors can be consistently detected in the adherent layer of long-term human marrow cultures for periods of at least 8 weeks. This is the first report of the persistence of this spectrum of primitive cell types after such prolonged periods of culture. The procedure used for establishing and maintaining cultures was essentially the same as that described by Gartner and Kaplan (7) and Greenberg et al (8) with the single proviso that retention of half the old medium at each weekly feeding was found to be markedly superior to a complete medium change. It should also be noted that in the present experiments, cultures were initiated with a single inoculum of cells from routine marrow aspirates rather than from rib or hip specimens. This latter point was of particular importance in the present study where we wished to obtain a data base for comparison with long-term cultures initiated with marrow cells from patients with CML or AML where only material from a single aspirate would be available.

Determination of the number of progenitor cells present in the adherent fraction necessitated the development of a suitable method for detaching and suspending the cells of interest. Simple mechanical detachment, for example using a rubber policeman, proved to be fraught with problems because of the numerous large clumps of cells obtained. On the other hand, satisfactory cell suspensions could be reproducibly obtained using either trypsin or collagenase and neither of these enzymatic treatments appeared to have significant adverse effects on progenitor cell plating efficiency. The collagenase treatment procedure developed was slow (3 hours incubation as compared to 10 min with trypsin), but had the advantage of more selective action. Thus, using collagenase, it was consistently possible to obtain final cell suspensions contain-

ing all detectable adherent layer progenitors but only 75% of all the total adherent layer cell population, whereas trypsin rapidly detached all cells indiscriminately. Although the collagenase preparation used was not pure, it seems reasonable to assume that part of its effectiveness was due to the breakdown of collagen. Collagen synthesis is known to occur in such cultures (19). The finding that collagenase could separate hemopoietic cells from some of the other components of the adherent layer suggests that collagen might be involved in intercellular connections between primitive hemopoietic cells and other adherent cell types with, as yet, poorly defined regulatory functions.

Recent studies of long-term mouse marrow cultures have established two important functions of the cells in the adherent layer. One is to modulate stem cell proliferation by the production of both positive and negative regulatory signals (20). The other is to provide the initial reservoir of stem cells from which all non-adherent cells are subsequently derived (2). The present experiments suggest that a similar flow of hemopoietic cells from the adherent to the non-adherent fraction occurs in long-term human marrow cultures. This has been since formally demonstrated by others (21). The key observation here was that the most primitive progenitor cell types, identified by their pluripotentiality or their very high proliferative capacity were always found preferentially, and at later times exclusively, in the adherent layer. Conversely, progenitors with restricted potentiality and lower proliferative capacity were more commonly found in the non-adherent fraction. This inequality in the type as well as the total number of progenitors present in the adherent and non-adherent fractions has several important implications. First, assessment of the non-adherent fraction only is likely to lead to a significant underestimate of the true progenitor content of the culture in a fashion that increases with time. Second, changes in the number of total

cells and progenitors in the non-adherent fraction observed each week are probably influenced by factors that affect the release of cells from the adherent layer. This would tend to invalidate the use of numerical corrections that assume the non-adherent cell population to be a self-sustaining compartment (22).

Finally, it should be noted that although many of the marrows used in the present study were from patients with lymphoid malignancies, 5 were from normal individuals (marrow transplant donors). Thus, the preferential retention of more primitive hemopoietic progenitor cell types in the adherent layer appears to be a consistent feature of long-term human marrow cultures in which hemopoiesis from normal progenitors is being maintained. This would suggest that assessment of the adherent layer could also be of importance in analyzing long-term marrow cultures from patients with leukemia since primitive normal stem cells, even if present, might not be expected to reach detectable levels in the non-adherent fraction.

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C H A P T E R I I I

LONG-TERM MARROW CULTURES FAVOUR THE PERSISTENCE OF Ph^1-NEGATIVE HEMOPOIETIC PROGENITORS IN CULTURES ESTABLISHED FROM PATIENTS WITH Ph^1-POSITIVE CML

1) INTRODUCTION

CML is a clonal disorder involving a myeloid pluripotent stem cell which acquires a tremendous growth advantage over normal hemopoiesis, but, during the chronic phase, retains its capacity to differentiate along pathways characteristic of normal hemopoiesis (1,2). In clonal assays, the amplification of the 3 lineages is detectable at the pluripotent (3) and committed stem cell level (4) and their leukemic origin has been demonstrated (5,6).

In Ph^1-positive patients, Ph^1-negative cells are usually not detectable. One explanation is that they are simply diluted to undetectable levels. However, it is possible also that the process of clonal expansion is associated with a suppression of normal stem cell activity leading initially to a reduction of their numbers and eventually to their extinction. Suppression of normal stem cells is also supported by studies of the effect of treatment. In most patients whose counts are brought under control with conventional therapy, chromosomally normal cells do not reappear. However, from the results of very aggressive treatment protocols usually resulting in high frequencies of transient cytogenetic conversion, it seems now likely that significant numbers of residual normal stem cells, although undetectable, may be present in many patients when their disease is first clinically recognized (7).

Long-term cultures offer an in vitro approach to the investigation of the mechanisms underlying normal and abnormal stem cell maintenance. Conditions

generated in long-term cultures are closer to the in vivo situation in that interactions between stromal and hematopoietic elements are likely to occur in the adherent layer. In support of that hypothesis, I have shown that pluripotent and primitive erythropoietic and granulopoietic progenitors would be consistently detected in such cultures for at least 8 weeks and that they were preferentially located in the adherent layer (see Chapter II).

To evaluate the usefulness of the long-term culture system for studying interactions between leukemic and normal cells, I set up long-term cultures with marrow cells from 11 patients with newly diagnosed Ph^1-positive CML and followed the kinetics of progenitor behavior in both the adherent and non-adherent fractions.

Because Ph^1-positive and Ph^1-negative progenitors cannot be distinguished by simple morphological assessment of the colonies they produce, cytogenetic analysis of individual colonies was performed to evaluate the relative numbers of leukemic and normal progenitors present in any given adherent layer assay. The results of these experiments are described below.

2) MATERIAL AND METHODS

(A) Patients

Clinical and hematological data on the 11 CML patients included in this study are summarized on Table V. There were 7 females and 4 men. The average age was 38, with a range of 12 to 63 years. For patients H.H. and S.McF., 2 marrow specimens were obtained 3 months and 1 month apart respectively. In both cases, no treatment was administered during this interval. Ten patients were recently diagnosed with no previous history of hematological problems and none had received chemotherapy prior to the time of study. One (S.M.) has been diagnosed 8 years previously and was on Busulfan therapy at the time of

TABLE V

Clinical and Hematological Data for the 11 CML Patients Included in this Study

Patient	Sex/Age	WBC (per mm^3)	Hb (g/dl)	Platelets (per mm^3)
L.A.	F/20	69,000	12	400,000
J.M.	F/54	21,000	13	400,000
H.Y.	F/37	130,000	11	670,000
H.H.-a*	M/30	12,700	14	400,000
-b*	M/30	30,000	14	400,000
R.McT.	F/35	86,000	12.5	370,000
P.D.	M/12	500,000	8.2	330,000
V.S.	M/49	46,000	15	230,000
S.McF.-a†	F/39	20,000	16	750,000
-b†	F/39	13,000	15	580,000
E.McG.	F/63	63,000	10	$>10^6$
J.L.	F/49	94,000	12	672,000
S.M.	M/33	48,700	14.7	340,000

* Two samples from this patient obtained 3 months apart.

† Two samples from this patient obtained 1 month apart.

study. In every case, the bone marrow aspirate and biopsy showed a typically increased cellularity and a high myeloid:erythroid ratio. Diagnosis was confirmed in all 11 patients by the presence of the standard Ph^1 translocation in direct marrow preparations (8,9). Marrow cells used for culture were part of aspirates taken for diagnostic purposes and were obtained with informed consent. Marrow for culture was collected in sterile tubes containing 800 units of preservative free heparin in 1 ml of α-medium.

(B) Establishment and Maintenance of Long-term Cultures

The procedure used to initiate and maintain long-term cultures has been described in detail in Chapter II (method A of initiation and method A of feeding). All 11 marrow specimens were very cellular ($1.19 \pm 0.19 \times 10^8$ cells per ml as compared to $0.53 \pm 0.4 \times 10^8$ cells per ml for control marrows - see Chapter II). When the concentration exceeded 1×10^8 cells per ml (7 patients), it proved necessary to first dilute the initial specimen so that the culture could be initiated with a more accurately evaluated inoculum. Cultures were incubated continuously at 33^oC in a humidified atmosphere of 5% CO_2 in air, except for 5 experiments (V.S., J.L., P.D., E.McG., and S.McF.), where cultures were incubated for the first 4 days at 37^oC and then transferred to 33^oC. Despite the very high initial cell concentration, red cell contamination was not negligible, and the day 3-4 separation step was performed by centrifugation of the non-adherent cells over 1.077 g/cm^3 Ficoll-Hypaque or 1.075 g/cm^3 Percoll. Light-density cells were then washed twice and returned to the cultures in 8 ml of fresh medium. For 3 patients (V.S., J.L., and P.D.), this step was skipped and all non-adherent cells were simply returned to the culture dish in 8 ml of fresh growth medium. All cultures were fed again on the 7th day after initiation, and thereafter at weekly intervals, as noted above.

The number of colony-forming progenitors present in the aliquot of marrow used to initiate long-term cultures was calculated from the number of colonies obtained in assays of the fresh marrow buffy-coat as described previously (see chapter II). The non-adherent cells removed each week were counted and also routinely assayed for colony-forming progenitors. To determine the progenitor content of the adherent fraction, cells were detached and suspended using collagenase. The procedure followed was the same as that described in Chapter II and was found to work equally well with long-term CML cultures. Previous studies with cultures of normal marrow showed that collagenase released only 75% of the total adherent cell population but detached all progenitors. The same observation was found with the CML cultures analyzed here.

(C) Hemopoietic Colony Assays

Erythropoietic (CFU-E and BFU-E), granulopoietic (CFU-C), and pluripotent (CFU-G/E) progenitors were assayed in methylcellulose cultures as previously described (chapter II). When cytogenetic analyses were planned, additional replicates were set up for this purpose. Fresh marrow buffy coat cells were plated at several concentrations between 2 and 0.2 x 10^5 cells per 1.1 ml assay to ensure that conditions were obtained where developing colonies did not overlap one another.

Numerical estimates of progenitor numbers were based on colonies scored in 2-4 assays. Adherent cells were plated routinely at 1 x 10^5 cells per 1.1 ml assay for both scoring and cytogenetic studies since the concentration of progenitors was never high enough to create overlap problems.

(D) Cytogenetic Methods

The general protocol used to distinguish different leukemic and normal populations is shown schematically in Figure 13. Three different types of preparations were analyzed cytogenetically in each experiment using solid Giemsa staining (G-banding (10)): (1) Direct marrow metaphases prepared following conventional methods (9), (2) metaphases from individual colonies produced in methylcellulose assays of the initial marrow specimen, and (3) metaphases from individual colonies produced in methylcellulose assays of adherent cells from long-term cultures terminated 2 to 8 weeks after initiation. The procedure for obtaining chromosome preparations from single large hemopoietic colonies was developed in the Terry Fox Laboratory (5). Each set of assay cultures was monitored at frequent intervals to select the optimal time for plucking i.e., when large colonies had matured to the point where they could be identified with confidence as erythroid, granulocytic or mixed, but colony growth had not yet stopped. This usually occured after 9-12 days for BFU-E and up to 15 days for CFU-C. As a result, different types of colonies were usually harvested at different times in different assay dishes. One hour prior to harvesting, 0.1 ml of Colcemid (1 ug/ml), was evenly distributed over the methylcellulose surface with a 26 gauge needle (7-8 drops). Selected colonies were then plucked, and dispersed in a small volume of 0.075 M KCL (20 ul/microwell). After 15-20 minutes in hypotonic medium, colonies were transferred one by one onto polylysine-coated slides and then fixed in a mixture of 3:1 methanol:acetic acid. Only colonies in which at least 2 metaphases could be analyzed were accepted. In the 280 individual colonies in this study that met that criterion, no example of more than one genotype per colony was encountered. The possibility of significant numbers of dividing background cells in the adherent layer assays was also ruled out

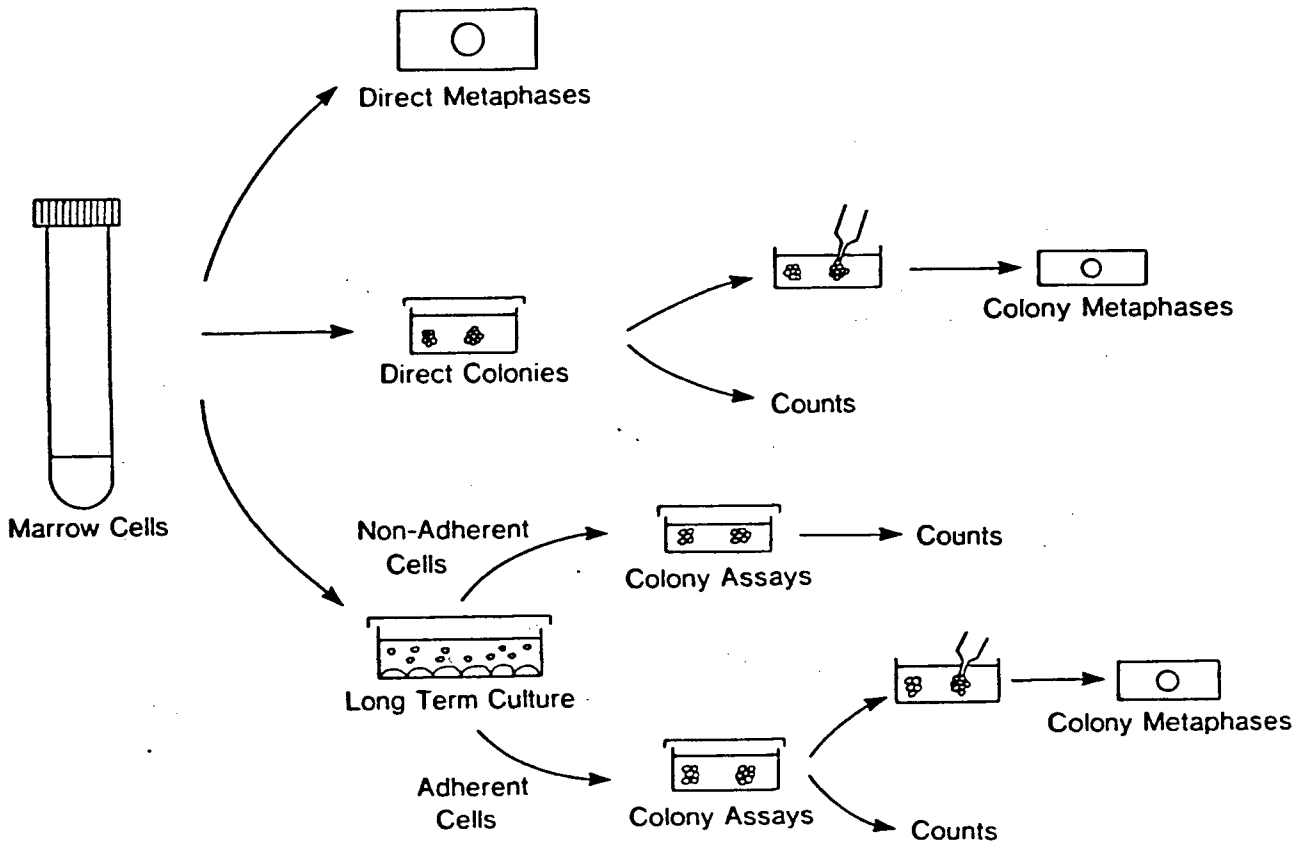

FIGURE 13. Protocol used to evaluate the phenotype and genotype of the progenitor populations detectable in fresh and cultured CML and AML marrow samples

by analysis of randomly chosen aliquots of intercolony regions. In such preparations, background metaphases were found to be rare.

3) RESULTS

(A) Hematopoietic Progenitor Cell Kinetics in Long-term CML Cultures

Long-term cultures were set up with marrow cells from 11 patients with newly diagnosed Ph^1-positive CML. Two patients (H.H. and S.McF.) were studied twice. From 2 to 7 replicate long-term cultures were set up from each marrow specimen. Thirty-five cultures were terminated at varying intervals up to 12 weeks after initiation to allow progenitor assessment of the adherent layer. As discussed in Chapter II, after 3-4 weeks the preferential location in the adherent layer of the most primitive hematopoietic progenitors (CFU-G/E, primitive BFU-E and high proliferative capacity CFU-C) was a key feature of normal long-term cultures. This unequal partitioning of primitive hemopoietic cells between the 2 fractions was also found to be a consistent feature of 12 of the 13 long-term culture experiments described here.

Nucleated Cell Content of the Adherent Layer. Visual inspection showed that typical adherent layers formed in the long-term cultures from 7 patients and measurement of the total cell content yielded values in the normal range. In all patients, confluence was reached at the same rate as in control cultures, and sometimes sooner, despite the possibility of dilution of stromal precursors by an expanded hematopoietic population. In the other 4 experiments (J.M., S.M., E.McG., and P.D.), the adherent layer did not achieve a normal confluent appearance (Figure 14), and its cellularity during the whole culture period remained below 1.5×10^6 cells. A mean value for all 35 CML cultures ended between weeks 2 and 12 was 1.8×10^6 cells (range $0.2 - 4.8 \times 10^6$ cells) as compared to $2.8 \pm 0.3 \times 10^6$ cells in the controls (see chapter

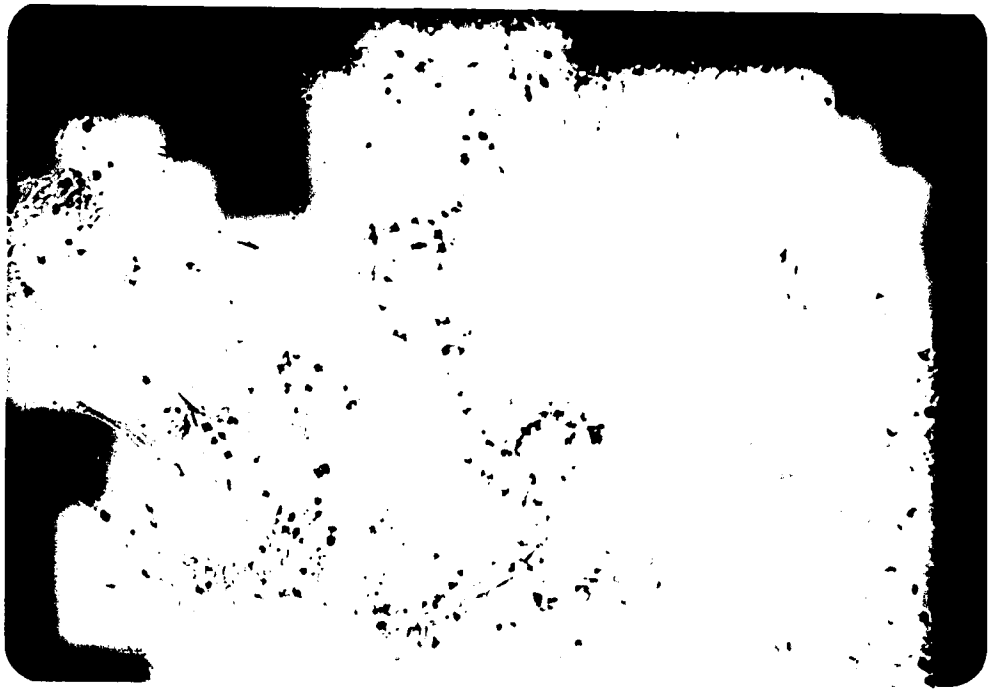

FIGURE 14. Abnormal appearing 5-week-old adherent layer from the long-term marrow culture of CML patient S.M. (x80)

II). As shown in Table VI, values stayed roughly constant during the culture period and were on average below normal controls at each time point. However, as just indicated, the interexperimental variation was greater than that seen with normal marrow samples. In 2 experiments (V.S., H.H.b), the number of adherent cells was markedly elevated (>4 x 10^6). The relevance of that observation to myelofibrosis characteristic of CML in vivo can only be speculated upon at this time.

Nucleated Cell Content of the Non-adherent Fraction. All long-term CML cultures showed differences from normal controls in their yield of non-adherent cells as a function of time (figure 15). The non-adherent fraction of CML cultures was found to undergo a rapid initial decline although in most of the cultures the onset of this decline was slightly delayed by comparison to normal controls. In addition, this decline typically continued for 2-3 weeks longer than normal and evidence of stabilization of this population was first apparent, if at all, only after 6-7 weeks.

Hemopoietic Progenitors Kinetics in the Adherent and Non-Adherent fractions. To evaluate the population dynamics of more primitive compartments that might help to explain the total nucleated cell findings, colony-assays for hemopoietic progenitors were performed. Non-adherent cells were assayed weekly. Adherent layers were assayed somewhat less frequently, since that required termination of the culture.

The non-adherent fraction data for 10 patients are shown on figures 16 and 17. Cultures from patients H.H. and S.McF., who were each studied on 2 separate occasions, gave similar results each time and therefore, data from only one experiment has been shown. On average, the progenitor content of the non-adherent fraction was found to be lower than that typical of normal cultures. BFU-E numbers were on average higher than normal in starting fresh

TABLE VI

Total Number of Cells in Long-Term CML Marrow Culture Adherent Layers (Released by Collagenase Treatment)

	Weeks in Culture			
	2	4	6	8
Patients				
Geometric mean (x 10^6)	1.59	2.05	1.81	1.82
Range (x 10^6)	0.23 - 4.8	1.3 - 3.5	1.1 - 3.9	1.05 - 3.5
No of cultures	9	10	5	4
Control				
Geometric mean (x 10^6)	2.9	2.8	3.2	2.5
Range (x 10^6)	2.3 - 3.7	2.4 - 3.1	2.6 - 4.0	2.4 - 2.7
No. of cultures	6	6	6	4

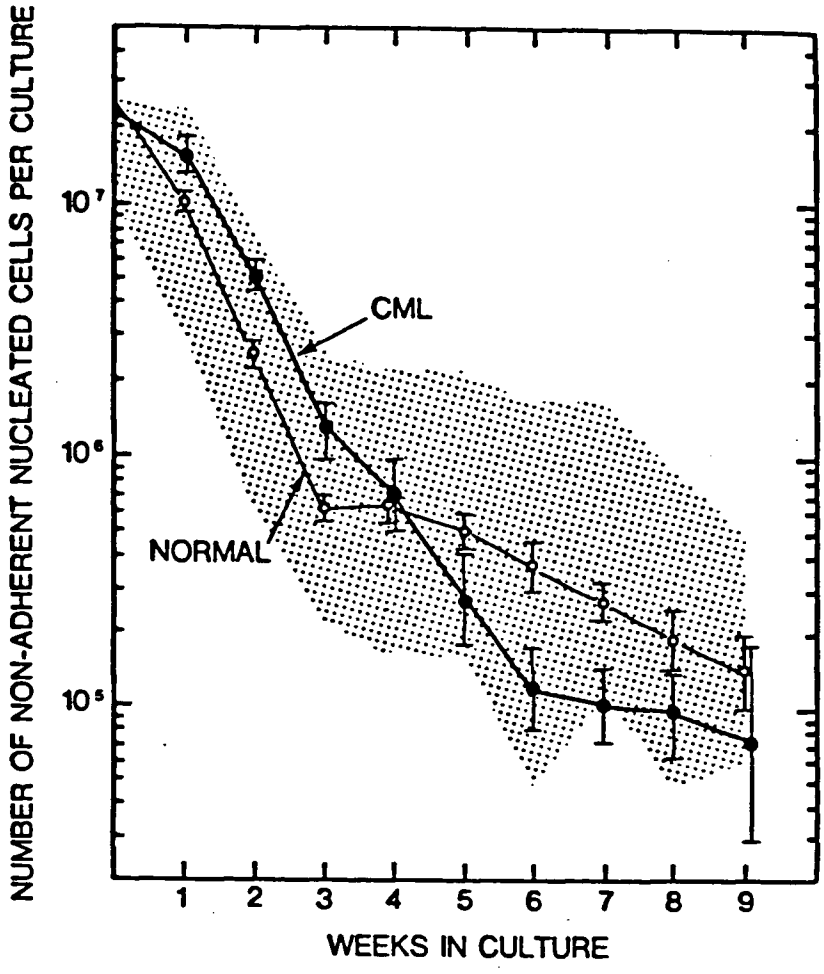

FIGURE 15. Total nucleated cell content of the non-adherent fraction in the 13 CML long-term experiments included in this study as compared with cultures from 32 normal marrows maintained under similar conditions. Each point indicates the geometric mean ± SEM. The shaded area shows the range of values obtained in control experiments established at the same time

marrow assays (Table VII), although not significantly so, and it is in this category that the decline in non-adherent progenitors was the most pronounced (figure 16). In 8 of the 13 cultures, the number of BFU-E fell below the limit of detectability by 4 weeks, and except for patients V.S. and S.McF., very few non-adherent BFU-E were seen thereafter. In some cultures, erythroid colonies appeared small, with a tendency to early lysis. BFU-E were, however, consistently detected in the adherent layer for periods of 6-8 weeks and in 2 experiments, BFU-E were still detectable at 9 (S.McF.), and 12 (V.S.) weeks (Table VIII). Nevertheless, the BFU-E content of the adherent layer was also reduced by comparison to normal cultures. The ratio of BFU-E detected in the adherent versus non-adherent fraction was on average 7 between week 2 and 4 and around 10 thereafter. Non-adherent CFU-C were consistently detected in the non-adherent fraction (Figure 17). CFU-C were partitioned between the adherent and the non-adherent fractions of CML long-term cultures in a fashion similar to that described for controls (Table VIII), i.e., the ratio of adherent to non-adherent CFU-C progenitors was below 1 (0.5) during the first 3 weeks, but increased to >3 after 4 weeks. However, the number of CFU-C was usually slightly lower than in control cultures of the same age. Thus, overall, the number of BFU-E and CFU-C in long-term cultures from patients with CML tended to be lower with a particularly marked decline in BFU-E numbers.

High proliferative CFU-C were present in most of the CML adherent layers processed (Figure 18) and in those CML cultures where CFU-C numbers were not severely decreased (i.e., H.H., V.S., and S.McF.), represented a similar proportion of the total adherent CFU-C population as was found in control cultures. Adherent as well as non-adherent colony-assays from these 3 experiments also gave results comparable to controls in terms of the lenght of time

TABLE VII

Number of Erythropoietic and Granulopoietic Progenitor Cells per 2 x 10^5 Buffy Coat Cells in the Starting Marrow of the 11 CML Patients Included in this Study

Patient	CFU-E	BFU-E	CFU-C
L.A.	246	127	25
J.M.	276	64	20
H.Y.	728	90	77
H.H.-a*	595	163	20
-b*	131	50	20
R.McT.	303	120	72
P.D.	126	55	500
V.S.	526	115	57
S.McF.-a†	476	125	26
-b†	770	135	40
E.McG.	1496	380	304
J.L.	328	96	24
S.M.	183	37	40
Controls			
Mean	107	70	64
Range	28-407	12-403	18-218

* Two samples from this patient obtained 3 months apart.

† Two samples from this patient obtained 1 month apart.

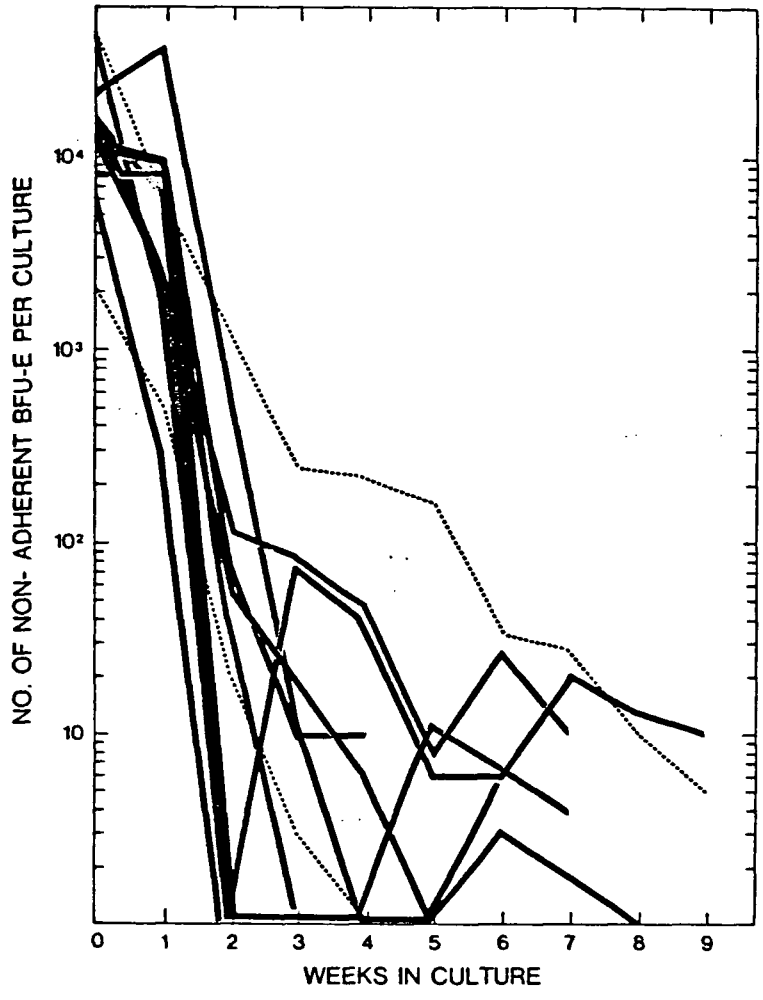

FIGURE 16. Number of non-adherent BFU-E as a function of time in long-term CML marrow cultures. Results are from 10 of the 11 CML patients (patient P.D. results not shown). Dotted lines indicate the corresponding range of BFU-E values measured in 22 normal long-term marrow cultures established at the same time

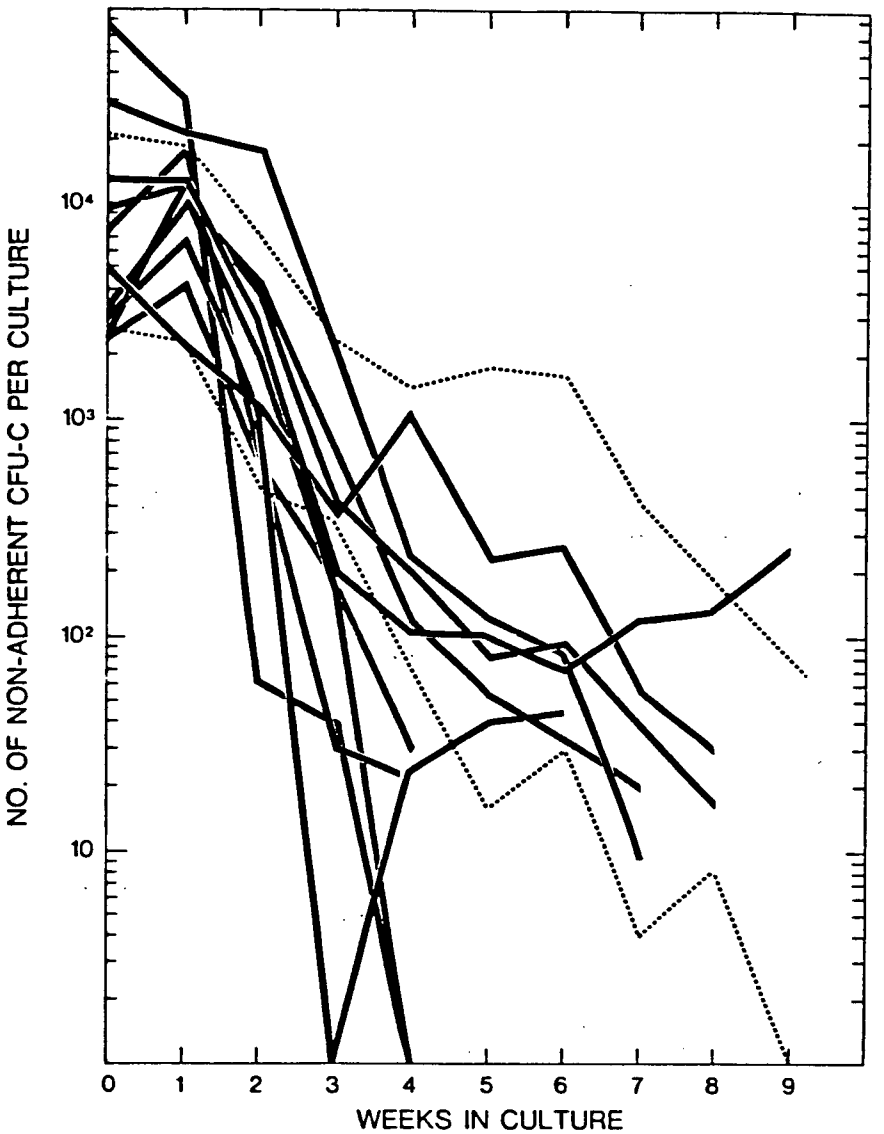

FIGURE 17. Number of non-adherent CFU-C as a function of time in CML long-term cultures. Results are from the same 10 patients as Figure 16. Dotted lines indicate the corresponding range of CFU-C values measured in 22 normal long-term marrow cultures established at the same time

TABLE VIII

Hematopoietic Progenitor Cell Content of the Non-Adherent (NA) and Adherent (A) Fractions of Long-Term Marrow Cultures from 2 CML Patients and from 31 Controls as a Function of Time in Culture

Patient	Duration of Culture (wks)	Progenitor No. (Total Per Culture)		
		BFU-E NA:A	CFU-C NA:A	CFU-G/E NA:A
V.S.	2	<55* : 214	1,788 : 3,740	<55* : 12
	3	72 : 235	192 : 916	<24* : 20
	4	14 : 115	316 : 763	<14* : 10
	6	9 : 331	205 : 1,884	<2* : <20*
	8	46 : 82	183 : 710	<3* : 4
	12	7 : 32	53 : 81	<3* : <3*
H.H.-a	2	30 : 250	4,060 : 1,029	<125* : 16
	4	5 : 53	134 : 326	<3* : 6
	6	ND : <3*	ND : 122	ND : <3*
	8	ND : <17*	ND : 52	ND : <17*
Controls†	2	127 : 446	2,250 : 2,328	6 : 46
	3	66 : 219	789 : 667	2 : 2
	4	79 : 340	682 : 1,142	2 : 4
	6	2 : 79	93 : 324	<2* : 3
	8	3 : 50	58 : 162	<2* : <12*

* Maximum value of one colony had been seen in any of the assay dishes scored.

† See Chapter II, Figures 10 and 12.

FIGURE 18. Large granulocytic colony observed in a 15 day old methylcellulose assay of a long-term CML marrow culture adherent layer (patient H.H.-a) (x80)

progenitors continued to be detected (up to 10 weeks). Only in these 3 patients were CFU-G/E detected, and in each of these cases, CFU-G/E were restricted to the adherent layer. Results obtained from adherent layer colony-assays after 6 weeks are not representative as only the 2 cultures where progenitors persisted were kept for longer periods.

(B) Cytogenetic Analysis of Progenitors Detected in CML Long-term Cultures

Cytogenetic studies were performed in 7 of the 13 experiments (H.H., S.McF., V.S., R.McT., and E.McG). Patients H.H. and S.McF. were studied twice. Analysis of individual hemopoietic colonies from adherent cell colony-assays showed a consistent shift from a predominantly Ph^1-positive to a predominantly Ph^1-negative progenitor population in 6 of the 7 experiments analyzed (Table IX).

The first patient (H.H.) was initially a mosaic (H.H.-a), with 5% normal metaphases in direct preparations (Table IX). Fourteen per cent of the colonies from fresh marrow assays that were analyzed were also Ph^1-negative. Analysis of colonies obtained from assays of long-term culture adherent cells after 4 weeks of incubation showed an overall increase in the proportion of Ph^1-negative progenitor cells to 53%. When this patient was studied again 3 months later, only Ph^1-positive metaphases were seen in the direct preparations (56 analyzed), and in all fresh marrow colonies (22 analyzed). Cytogenetic data were obtained from 22 colonies generated in assays of 2 to 6 week-old long-term culture adherent layers. Of these, 14 colonies (67%) were normal and only 7 colonies (33%) were Ph^1-positive. As can be seen in Figure 19, normal progenitor cells in this experiment were already detectable in 2 week-old adherent layers and became the predominant type present by 4 weeks.

TABLE IX

Results of Cytogenetic Analysis of Direct Marrow Preparations and Hemopoietic Colonies Generated in Methylcellulose Assays of the Initial Marrow and 1 to 8 Week-Old Long-Term Culture Adherent Layers (LTC-AL) from 5 CML Patients

Patient	Initial Marrow		LTC-AL Colony Assays			
	Direct Preparation	Colony Assays	BFU-E	CFU-C	CFU-G/E	(Wks)
H.H.-a†	5/101*	3/21	2/4	7/13	—	(4)
H.H.-b†	0/44	0/22	10/11	4/10	—	(2-6)
S.McF.-a^{O}	0/30	3/10	11/11	1/1	—	(4-6)
S.McF.-b^{O}	0/13	7/24	10/10	—	—	(1)
R.McT.	0/33	0/27	3/3	2/2	—	(4)
V.S.	0/43	0/15	52/57	6/9	1/1	(2-8)
E.McG.	0/20	0/4	0/25	—	—	(3-4)

* Values shown are the number of Ph^{1}-negative cells or colonies expressed as a fraction of the total number analyzed.

† Two samples from this patient obtained 3 months apart.

^{O} Two samples from this patient obtained 1 month apart.

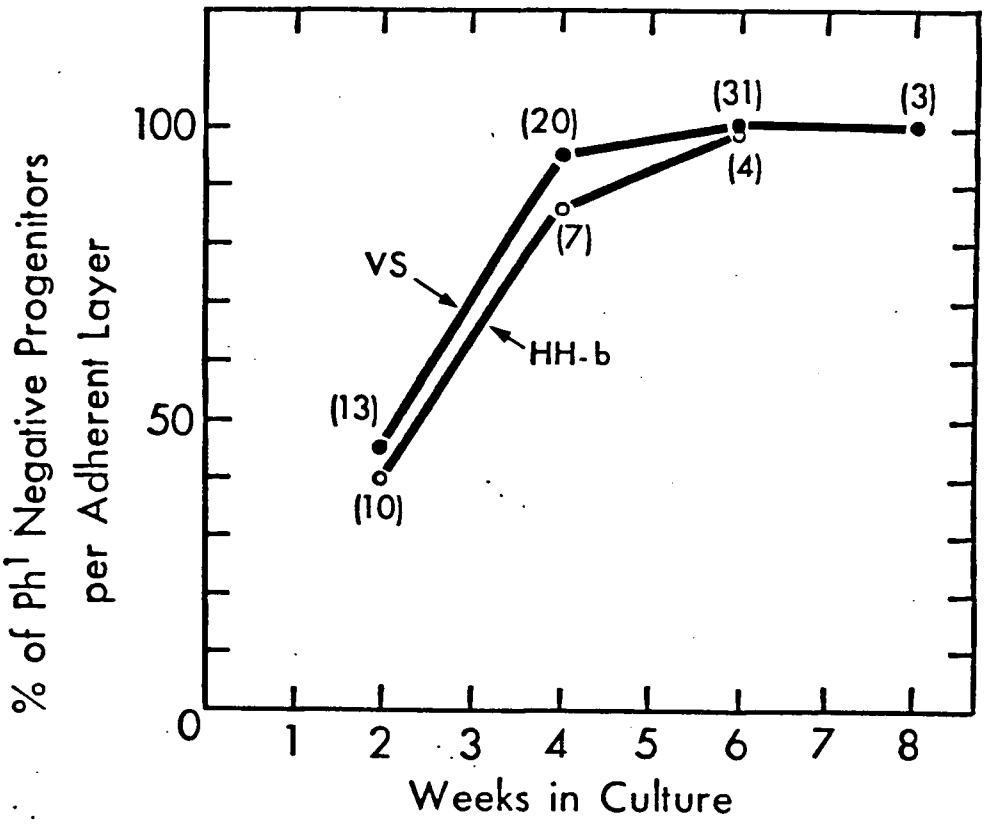

FIGURE 19. Time-course studies of the appearance of Ph^1-negative progenitors in 2 long-term CML marrow cultures. Each point represents the percentage of all colonies analyzed that were Ph^1-negative. Figures in parentheses refer to the total number of colonies analyzed at each time point

Interestingly, by 2 weeks, 4 of the 5 BFU-E analyzed were Ph^1-negative whereas all the 5 CFU-C analyzed were Ph^1-positive.

The next patient with newly diagnosed Ph^1-positive CML was also studied twice one month apart, and each time showed a detectable level of mosaicism in fresh marrow colony assays but not in the direct marrow metaphase preparation. This decrease in mosaicism between the progenitor and terminally differentiating (direct marrow) compartments has been documented in several other CML patients. For this patient, 22 colonies from adherent layer assays were analyzed at week 1, 4 and 6 and all were found to be Ph^1-negative. Also it should be noted that after only 1 week in culture, only Ph^1-negative BFU-E were detected in the adherent layer.

Patients 3 and 4 (V.S., and R.McT.) with newly diagnosed Ph^1-positive CML and no detectable mosaicism gave similar results. In both cases, only Ph^1-positive progenitors were detected after analysis of colonies from fresh marrow assays. However, after 4 weeks in long-term culture, most of the progenitors in the adherent layer that yielded analyzable colonies were consistently normal (Table IX). In one of these experiments (V.S.), colonies from 2 week-old adherent layers were also available for cytogenetic analysis and revealed readily detectable Ph^1-negative progenitor cells (figure 19). In this case, the same ratio of Ph^1-negative and Ph^1-positive cells were found in both CFU-C (3 out of 6) and BFU-E (4 out of 7) compartments.

The fourth patient with newly diagnosed Ph^1-positive CML (E.McG), showed no detectable mosaicism either in direct marrow preparations or in fresh marrow erythroid or granulocytic colonies. Long-term cultures in this case showed reduced numbers of primitive BFU-E and CFU-C in adherent layer assays and all 25 adherent layer colonies analyzed proved in this case to be Ph^1-positive.

The majority of the colonies analyzed cytogenetically were large erythropoietic colonies containing more than 8 clusters of erythroblasts. These were preferentially selected because of the negligible chance of misidentification and they commonly yielded 2 or more suitable metaphases. In the 2 experiments (H.H.-b and V.S.) where cytogenetic analysis was used to evaluate adherent layer progenitors after varying periods of long-term culture (figures 19 and 20), a sufficient number of erythroid colonies were analyzed at each point to enable total numbers of Ph^1-positive and Ph^1-negative primitive BFU-E (>8 clusters) in the adherent layer to be estimated. The results for V.S. are shown on figure 20. It can be seen that the Ph^1-positive population disappeared with very rapid kinetics, whereas the Ph^1-negative population was maintained at a relatively constant level throughout the 4-8 weeks period of observation. A similar pattern was observed for the BFU-E from patient H.H.-b. (data not shown). Table X shows the pattern of changes in primitive Ph^1-negative and positive BFU-E for all 5 experiments. This emphazises the abnormally rapid decline in Ph^1-positive BFU-E in all 5 experiments, whereas Ph^1-negative BFU-E kinetics, in the 4 experiments where they were detected, did not differ greatly from that seen in normal long-term marrow cultures considering the initial input of Ph^1 negative progenitors.

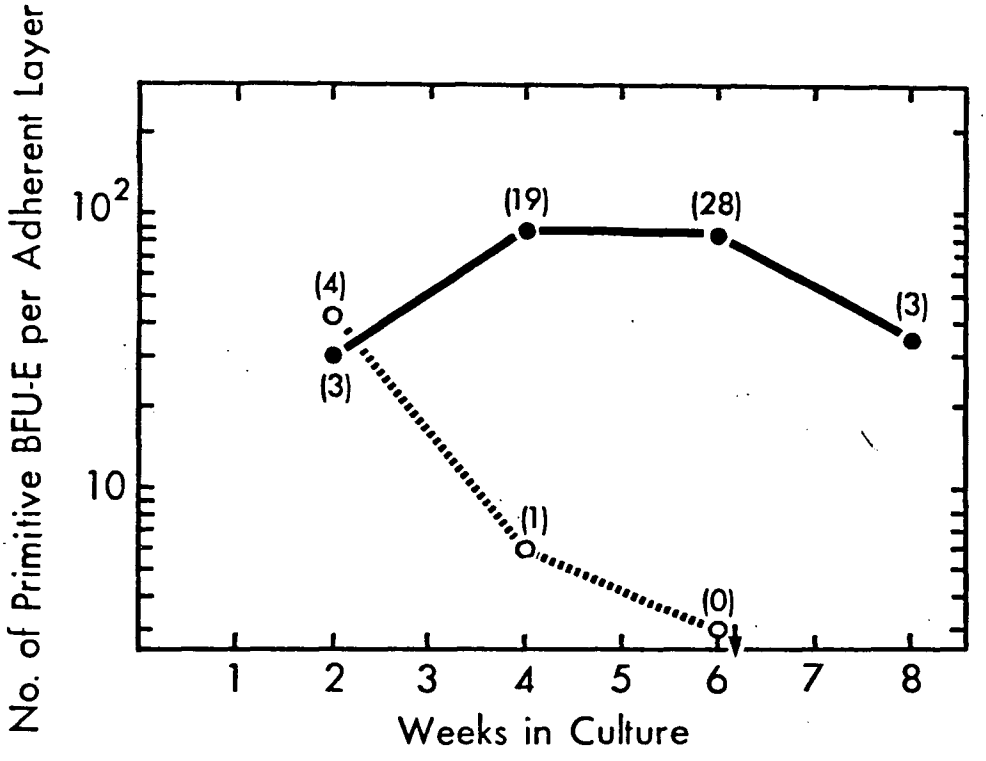

FIGURE 20. Absolute numbers of Ph^1-negative (●) and Ph^1-positive (O) primitive BFU-E present in the adherent layer of long-term marrow cultures from patient V.S. after various periods of culture maintenance. Figures in parentheses refer to the number of large erythroid colonies analyzed cytogenetically at each time point

TABLE X

Number of Ph^1-Positive and Ph^1-Negative Primitive BFU-E in the Adherent Layer of 5 Long-Term CML Cultures*

	Ph^1-Positive			Ph^1-Negative		
Duration of Culture (wks)	0	3-4	6	0	3-4	6
Patient						
H.H.-b	7750	<17	<25	<930	17	25
R.McT.	1250	<25	--	<225	25	--
V.S.	1500	6	<36	<390	109	331
S.McF.-a	2100	<17	--	900	71	--
E.McG.	1500	55	--	<615	<13	--

* Values were estimated by multiplying the total number of primitive BFU-E per adherent layer by the percent determined to be Ph^1-positive (or negative). When none of a particular genotype were detected, maximum values were calculated. In these instances, the percent value used was the maximum that could be excluded with 95% confidence according to the number of colonies analyzed.

4) DISCUSSION

At presentation, more than 80% of patients with Ph^1-positive CML fail to exhibit detectable mosaicism in direct preparations of their dividing marrow cells (11,12). Although Ph^1-negative cells may sometimes be present at a higher frequency in more primitive committed progenitor compartments, as found here for patient H.H. and S.McF., persistence of mosaicism at the level of progenitor cells has not been a common feature in CML (4,13). Nevertheless, the results of Goto et al have shown that aggressive chemotherapy initiated soon after diagnosis can frequently induce transient repopulation of the marrow with Ph^1-negative cells (14). This indicates that significant numbers of functional but suppressed Ph^1-negative stem cells are still present in many patients at the time of diagnosis. The experiments described here show that it is possible to detect such cells using long-term cultures. It appears that Ph^1-negative primitive progenitors, like their counterparts in normal marrows, can be maintained for several weeks in the adherent layer of long-term marrow cultures. In contrast, the Ph^1-positive population rapidly declines. Because of this differential behavior, Ph^1-negative progenitors, even when present at undetectable levels in initial marrow specimens, may thus become readily demonstrable within 2 to 4 weeks in culture. This proved to be the case for 4 of the 5 such marrows studied here and in all 4 cases, the proportion of Ph^1-negative progenitors reached 100% by 4 to 6 weeks.

It seems unlikely that the predominance of Ph^1-negative progenitors observed after culture was significantly influenced by technical considerations, since no difficulty was encountered in detecting Ph^1-positive colonies in fresh marrow assays from the same patients. Moreover, in the case of patient H.H., where some Ph^1-negative cells were found in direct preparations, the proportion of Ph^1-negative progenitors (mainly BFU-E) detected in the same

initial marrow specimen was only slightly higher (13% versus 5%). In addition, we have recently found evidence for the occurence of a similar selective process in the adherent layer of cultures initiated with marrow cells from patients with other types of clonal hemopathies. These include several cases of AML (see Chapter IV) where the disappearance of abnormal clusters and colony-forming cells allowed the presence of normal progenitors to be detected. Similarly, in one case of P.V., we found that erythropoietin-dependent BFU-E persisted for a longer period than did erythropoietin-independent BFU-E (Coulombel, unpublished observations).

At present, little is known about the basis of the differential behavior of "normal" and leukemic cells in such cultures. One possibility is that many leukemic stem cells are poorly adherent and therefore compromised in their ability to become established in the adherent layer where survival may be enhanced. The results obtained when S.McF.'s cultures were analyzed only 7 days after initiation would tend to support such a hypothesis, since selection in the adherent fraction was already clearcut at this time. Nevertheless, alternative explanations (e.g. different requirements of neoplastic stem cells, or relatively fewer of these in the marrow in spite of high progenitor numbers) cannot yet be ruled out.

Whether or not the Ph^1-negative progenitors revealed in long-term CML marrow cultures represent residual normal cells also remains to be established. In the female G6PD heterozygote with CML studied by Singer et al, reappearance of nonclonal Ph^1-negative cells after aggressive chemotherapy was demonstrated (15), and very recently, evidence for nonclonal Ph^1-negative progenitors revealed in one long-term CML culture has been obtained (16). On the other hand, since it has been suggested that the acquisition of the Ph^1 chromosome may occur after clonal expansion is already underway (17,18), it is

posssible that long-term marrow culture might provide a favourable environment for their selection.

In one of the 2 experiments where time course cytogenetic studies were performed, there was no discrepancy in the proportions of Ph^1-negative BFU-E and CFU-C measured after 2 weeks when these progenitors were first seen. However, in the other long-term experiment, the majority of the BFU-E were Ph^1-negative after 2 weeks, whereas in the same assay, all granulopoietic colonies analyzed were Ph^1-positive. Although this latter finding is based on small numbers of both types of colonies, it suggests that Ph^1-positive cells may tend to persist for longer periods in the CFU-C compartment. A similar finding was noted in studies of the relative rate with which nonclonal BFU-E and CFU-C reappeared in the aggressively treated CML patient studied by Singer et al (19). Clearly it will be necessary to follow a larger series of cultures to establish whether Ph^1-negative CFU-C are commonly detectable as early as or later than Ph^1-negative BFU-E.

Cytogenetic findings may also help to understand the maintenance pattern of primitive progenitors in long-term cultures. The finding of low numbers of nucleated cells and hemopoietic progenitor cells in both the adherent and non-adherent fractions after 4 weeks can be explained if we speculate that progenitors located in the non-adherent fraction were Ph^1-positive at the beginning of the culture and Ph^1-negative thereafter. Granulocytic and erythroblastic colonies generated in the non-adherent cell assays were too small, (100-500 cells per colony), to be individually analyzed cytogenetically. We can assume that the abnormally rapid decline of their numbers in the non-adherent fraction parallels their disappearance from the adherent layer. Even though Ph^1-negative progenitors were easily detectable and preferentially maintained, they were not present in the initial marrow aliquot in numbers high enough to

repopulate the culture once the Ph^1-positive cells had disappeared, except in rare cases, such as V.S. in this study. If this is correct, one would expect to find exclusively Ph^1-positive cells in the non-adherent fraction during the first weeks and later on, Ph^1-negative cells released from the adherent layer.

In one of the 5 patients (E.McG.), Ph^1-negative progenitors were not detected in adherent cell assays. One possible explanation for this finding was that the initial marrow sample was inadequate in its ability to generate a competent adherent layer-supportive structure. Such a hypothesis was supported by the finding that the adherent layer remained patchy and did not reached confluence. Previous studies with both human and mouse long-term cultures have indicated that the majority of the most primitive pluripotent stem cells are preferentially retained in the adherent layer embedded in a confluent matrix of non-hemopoietic elements (20). Although the role of the non-hemopoietic component is not yet known, there is some evidence to suggest that if these cells are defective, long-term stem cell maintenance will be adversely affected (21,22). Thus, the correlated lack of both primitive and Ph^1-negative progenitors in the adherent layer of E.McG.'s cultures might have been due to such a defect. On the other hand, a greatly reduced input of Ph^1-negative stem cells might also have helped to explain the absence of detectable Ph^1-negative progenitors in this patient's cultures.

In the past decade, several attempts have been made to establish whether the existence or induction of mosaicism might be associated with longer survival (23). In general, the findings have not provided strong support for such an hypothesis and chemoradiotherapy with rescue of marrow failure by transplantation seems to be the only procedure that consistently restores normal hemopoiesis for significant periods of time (24,25). However, bone marrow transplantation is not an option for many patients. The differential kinetics

of Ph^1-negative and Ph^1-positive hemopoietic populations in long-term cultures described here suggest a new approach to the investigation of profound differences in normal and abnormal stem cell regulation that might eventually be exploited to therapeutic advantage. In addition, long-term cultures may provide a method for identifying patients with significant numbers of persisting Ph^1-negative stem cells, in particular when these remain below the limit of detectability by direct assays.

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C H A P T E R I V

LONG-TERM MARROW CULTURE OF CELLS FROM PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA (AML): SELECTION IN FAVOR OF NORMAL PHENOTYPES IN SOME BUT NOT ALL CASES

1) INTRODUCTION

Acute leukemia is characterized by the appearance in the marrow and blood of a large population of cells that fail to differentiate normally. It is now clear that these represent the clonal progeny of a single transformed cell, although recent evidence indicates that, at least in some patients, this cell may retain to varying degrees the ability to generate apparently normal blood elements (1). Studies with short-term clonogenic systems suggest the existence within the abnormal clone of progenitor populations of greater and lesser proliferative ability, analogous in this respect to the various subclasses of progenitor cells that normally maintain blood cell production (2-4). However, little is known about the contribution of these blast cell progenitors to clonal amplification in vivo or how they may suppress the proliferation and differentiation of persisting normal (nonclonal) progenitors.

To approach these questions, I explored the potential of the long-term marrow culture system since earlier studies had shown that primitive pluripotent and committed progenitors could be maintained under these conditions for periods of 8 weeks or more in cultures established from mouse (5,6) as well as human (7,8 and chapter II) marrow cells.

This chapter describes my experience with long-term cultures initiated with marrow from newly diagnosed AML patients and maintained for a minimum period of 6 weeks. The findings obtained refer to a series of 13 consecutive-

ly accrued patients for whom, in all but 2 initial cases, sufficient marrow cells were available to establish at least 1 long-term marrow culture (i.e., $>2 \times 10^7$ cells).

2) MATERIALS AND METHODS

(A) Patients

The clinical and hematological data for the 13 AML cases studied are shown in Table XI. For convenience, they have been grouped according to whether normal granulocyte colony-forming progenitors were detectable in long-term cultures that had been maintained for more than 4 weeks (patients 1-9) or not (patients 10-13). In each of these groups, those cases where progenitors of abnormal (blast) colonies were initially detectable are listed first. Eight of the 13 patients were females; the other 5 were males. The average age was 58 years (with a range of 7 to 80). All 5 types of disease according to the FAB classification (9) are represented. Marrow specimens from 12 of the 13 patients were taken prior to the initiation of any treatment. The marrow from patient J.O. was taken after one course of chemotherapy when the peripheral blood still showed 60% blasts. In 3 patients (A.McN., R.C., and J.O.) transformation from a preleukemic phase was suspected on the basis of the histological appearance of the marrow.

Marrow specimens were part of aspirates taken for diagnostic purposes and were obtained with informed consent. Each aspirate was collected in a sterile tube containing 800 units of preservative-free heparin in 1 ml of α-medium

(B) Establishment and Maintenance of Long-term Cultures

The procedure used to initiate and maintain long-term marrow cultures was the same as that described in chapter II (method A of initiation and method A

TABLE XI

Clinical and Hematological Data for the 13 Patients with Newly Diagnosed AML Included in this Study

Patient	Sex	Age	Peripheral Blood				Bone Marrow	
			Hg (g/dl)	WBC (per mm^3)	Blasts (%)	Platelets (per mm^3)	Blasts (%)	Fab Classification
J.B.	F	60	12	1,600	9	160,000	41	M2
T.E.	F	34	11	8,200	12	122,000	35	M2
J.R.	M	28	9	218,000	87	91,000	87	M4
A.McN.	M	67	6	3,400	23	7,000	35	Not classified
P.B.	M	63	8	380,000	97	75,000	82	M5
H.M.	F	70	9.4	700	0	83,000	65	M3
H.B.	F	80	13	14,800	3	180,000	40	M4
K.M.	M	62	6	1,600	5	157,000	35	M2
R.B.	F	7	6	15,000	51	14,000	90	M1
R.C.	F	72	8	5,600	6	6,000	40	M2
L.H.	F	74	8	28,000	96	36,000	97	M1
J.O.*	M	57	8	7,100	60	58,000	44	Not classified
E.T.	F	79	7.3	10,000	10	60,000	47	M2

* Marrow from this patient was obtained after one course of chemotherapy.

of feeding). In most experiments, cultures were incubated at 37°C for the first 3-4 days and then subsequently at 33°C. Red cells and granulocytes were usually removed on day 3 or 4 by centrifuging the total non-adherent fraction on Ficoll-Hypaque (1.077 g/cm^3, LSM, Litton Bionetics) or Percoll (1.075 g/cm^3, Pharmacia, Sweden) and returning only the light-density cells to the cultures. For 3 patients: P.B., R.C., and L.H., red cell contamination of the initial marrow was minimal and this step was omitted. On day 7 and at weekly intervals thereafter, cultures were fed by a complete change of the medium. At the same time, half of the non-adherent cells were also removed (method A of feeding, see chapter II).

The total cellularity and progenitor content of the non-adherent fraction was determined weekly. For 4 patients, cytospin preparations of the non-adherent cells were also made weekly and detailed morphology assessment of at least 300 cells carried out after staining with May-Grunwald-Giemsa. Differential was performed on a minimum of 300 cells. The adherent layer was assessed less frequently, since this required termination of the culture. Adherent cells were detached using 0.1% collagenase as described previously (see chapter II). As for normal and CML cultures, this procedure allowed all progenitors to be suspended, although approximately 25% of the other cell types in the adherent layer were not detached.

For 2 patients (J.B. and K.M.) the initial marrow aspirate contained less than 1.5×10^7 cells per ml. Earlier experience had indicated that cultures initiated with normal marrow samples of such cellularity yielded scanty adherent layers in which long-term maintenance of progenitors was poor. However, these two hypocellular marrow samples were included because it was felt desirable to survey initially all possible types of AML. To facilitate maintenance of hemopoietic cells, marrow cells from these two patients were

added to dishes containing pre-established marrow fibroblast "feeders" in the hope that these might compensate for a lack of such cells in the hypocellular aspirates. Feeders were obtained by subculturing primary normal marrow adherent layers 3 times using α-medium plus 20% FCS but without other supplements (including horse serum and hydrocortisone) in an attempt to eliminate residual normal hematopoietic progenitors as shown by control determinations (data not shown). Cultures established on such feeders were subsequently handled as usual.

(C) Colony Assay Procedure

Erythropoietic (CFU-E and BFU-E), granulopoietic (CFU-C), and pluripotent (CFU-G/E) progenitors were assayed as previously described in chapter II. Burst-promoting and colony-stimulating activities were provided by adding in the culture either agar-stimulated human leucocyte-conditioned medium (A-LCM,(10)) or PHA-stimulated human leucocyte-conditioned medium (PHA-LCM,(11)). A-LCM was sometimes used in initial assays of the original marrow buffy-coat at a final concentration of 9%. In all other instances, PHA-LCM was used at a final concentration of 4.5%. Both types of LCM's were pretested and calibrated against a standard preparation and found to be equivalent in their ability to support normal and abnormal colony growth in methylcellulose assays.

Fresh marrow cells were assayed by plating 2×10^5 buffy-coat cells per 1.1 ml of culture. Cells from the non-adherent and adherent fractions were usually plated at a final concentration of 1×10^5 cells per 1.1 ml of culture. When cytogenetic studies were planned, additional assay replicates were set up (see chapter III).

Dishes were scored twice, first after 9-12 days and again after 17-21 days of incubation as for normal specimens (see chapter II). However, scoring

criteria had to be modified due to the occurence of abnormal growth patterns in AML colony-assays. Thus, in those assays where morphologically abnormal (blast) clusters and/or larger colonies were obtained, accurate enumeration of morphologically normal but small granulocyte colonies that may also have been present was usually not feasible. Assessment of CFU-G/E in such assay cultures was also compromised. Detailed morphological studies of abnormal colonies and clusters were not undertaken, but periodic plucking and staining with May-Grunwald-Giemsa confirmed their blast cell nature as described by others (2-4). Because of the sometimes diffuse colony morphology, the often highly variable size of these abnormal clusters and colonies, and the persistence of a variable background of blasts in assays of different leukemic marrows, accurate quantitation of abnormal clusters and colonies was also usually difficult. In the present study, we therefore adopted the following semi-quantitative categories: "-" -- no evidence of abnormal (blast) colonies or clusters, "+" -- up to 50 abnormal colonies and/or clusters per assay (2×10^5 initial marrow buffy coat cells or 10^5 long-term culture cells), "++" -- 50 to 500 abnormal colonies and/or clusters per assay, "+++" -- >500 abnormal colonies and/or clusters per assay.

(D) Cytogenetic Studies

Direct preparations of Giemsa-banded marrow metaphases were obtained using standard methods (12). The genotype of primitive progenitors present in long-term marrow cultures was determined by analyzing metaphases from individual large colonies generated in methylcellulose assays of adherent layer cells. Colonies to be analyzed were selected and processed as described (chapter III and (13)). A minimum of 2 metaphases per colony were analyzed after G-banding and in none of the 34 colonies that satisfied this criterion was more than one genotype evident.

Cytogenetic analysis of fresh marrow progenitors was not attempted because of the anticipated very low incidence of morphologically normal colonies of suitable size and the frequent high background of abnormal growth in initial assays (e.g., see Table XIII).

3) RESULTS

(A) AML Long-term Culture Morphology.

In 10 of the 11 experiments where long-term marrow cultures were initiated in the standard way, a typical confluent adherent layer formed in which "cobblestone" areas of hemopoietic cells could be consistently discerned (Figure 21A). The establishment of the adherent layer was not delayed in these cultures in spite of the expected dilution of stromal precursors by an amplified blast population. One experiment (R.C.) showed a higher than normal nucleated cell content of the adherent layer (>4 x 10^6 cells). The 2 cultures initiated by adding AML marrow cells to pre-established normal "feeders" (see Materials and Methods) developed a similar appearance to the other 11 where feeders were not used. In one case (L.H.), only a partial adherent layer formed and confluence was not achieved (Figure 21B).

(B) Nucleated Cell Recovery in AML Long-term Culture Adherent and Non-adherent Fractions

The average total number of nucleated cells present in the non-adherent fraction of all 13 experiments as a function of time is shown in Figure 22. The results appear similar to those for control marrow cultures, at least for the first 6 or 7 weeks. However, as a group the AML cultures displayed

(A)

(B)

FIGURE 21. Morphological appearance of long-term AML marrow culture adherent layer:
(A) a 5 week-old confluent adherent layer with a typical cobblestone area (culture from patient H.B.) (x80)
(B) a 5 week-old abnormal-looking adherent layer (culture from patient L.H.) (x80)

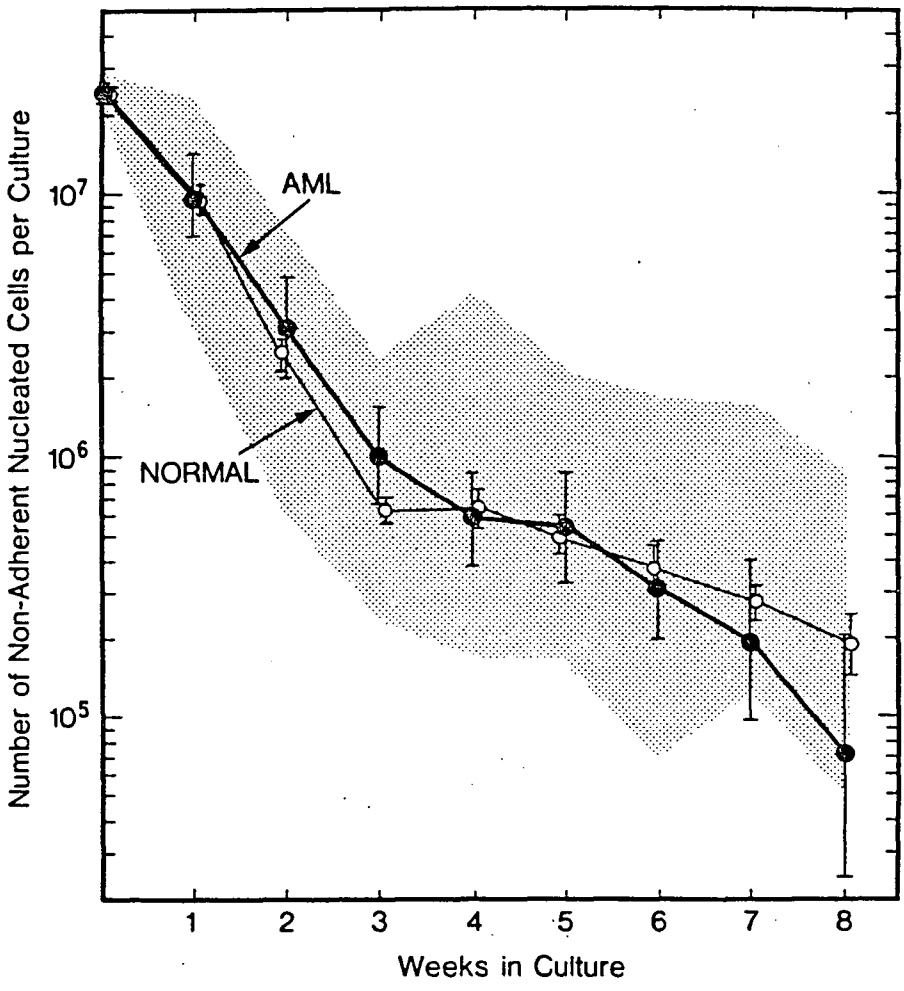


FIGURE 22. Total cellularity of the non-adherent fraction in the 13 long-term AML marrow cultures included in this study, compared with 32 normal marrows maintained under similar conditions. Each point indicates the geometric mean ± SEM and the shaded area the range of control values

greater heterogeneity. For example, in 3 (R.C., L.H., E.T.), non-adherent cell numbers were maintained between weeks 3 and 6 at levels higher than the maximum measured in any of 38 normal marrow cultures (see chapter II) during the same interval (i.e., $>2\text{-}4 \times 10^6$ non-adherent cells per culture). On the other hand, in another AML culture (R.B.), the non-adherent nucleated cell count fell to abnormally low levels ($<10^5$ cells) within the first 2 weeks, and never recovered.

Morphological examination of May-Grunwald-Giemsa stained cytospun preparations of the non-adherent cells from several of the cultures showed that blasts were a common cell type in the non-adherent fraction for the first 4 weeks (although with frequent cytological evidence of lysis) and blasts frequently usually persisted for several additional weeks (patient E.T.). Evidence of terminally differentiating granulocytes or monocyte/macrophages were also obtained in all instances, although these were sometimes clearly abnormal,e.g., as shown by the presence of Auer rods (see Table XII). Data from 4 experiments (patients P.B., H.M., R.C., and E.T.) are shown in Table XII.

The number of cells harvested from the adherent layer by collagenase treatment was determined between weeks 4 and 9 in 10 experiments. In general, the values obtained (mean = 2.7×10^6 cells, range = $1.7\text{-}5.4 \times 10^6$ cells) were similar to control cultures (mean = 2.8×10^6 cells, range = $0.8\text{-}6.3 \times 10^6$ cells, see chapter II)) with, however, much wider variations. The 2 cultures initiated on pre-established feeders were not included in these assessments.

TABLE XII

Morphological Analysis of the Non-Adherent Fraction of 4 Long-Term AML Marrow Cultures as a Function of Time after Initiation

Patient	Weeks of Culture	Differential (% values based on 300 cells analyzed)		
		Blasts	Myelocytes, metamyelocytes and polymorphs	Others° (mainly monocytes and macrophages)
P.B.	1	52	0	48
	2	60	6	34
	4	52	12	36
H.M.	1	84	<2%	15
	2	93	"	5
	3	96	"	3
	4	94	"	4
	5	86	"	13
R.C.	1	66	20	14
	2	57	30	13
	3	74	23	3
	4	47	46	7
	5	20	68	12
	6	19	72	9
E.T.	1	38*	44	18
	2	51*	22*	27
	3	60*	21	19
	4	72	25	3
	5	37	31	32

°Including some erythroblasts for the first 2 weeks only, and occasional lymphocytes (<5%), and usually <1% of cells that could not be identified.

*Presence of Auer Rods noted.

(C) Progenitor Cell Kinetics in the Non-adherent and Adherent fractions of Long-term AML Marrow Cultures.

Behavior of Progenitors of Abnormal Clusters and Colonies. For 8 of the 13 patients, abnormal (blast) colonies and/or clusters were readily apparent in initial marrow assays (e.g., see Figure 23 and Table XIII). In 2 other cases (H.B. and K.M.), these became evident in assays of the non-adherent fraction of 1-week-old long-term cultures, even though such colonies and/or clusters were not detected in assays of the original marrow sample (Table XIII). Thus, for 10 of the 13 patients studied, marrow cells capable of producing abnormal colonies or clusters were detected during the first 1-2 weeks in long-term cultures. These decreased progressively with time in long-term culture in 8 cases (Table XIV). In one of these (H.M.), such cells were no longer found after 1 week. However, in the other 7 cultures they persisted at detectable levels for 3 weeks and occasionally for 4 weeks, but not thereafter (Table XIV). Adherent layers from these long-term cultures, which were usually evaluated first after 4 weeks, also did not contain any detectable progenitors of abnormal (blast) colonies or clusters by this time (Table XV). The few (<20 per dish) abnormal clusters detected in colony-assays from 4 week-old adherent cell layers of 2 patients (T.E., and J.R.) did not impair detection of normal progenitors (see later).

A contrasting pattern was seen in long-term cultures initiated with marrow cells from the other 2 patients where abnormal (blast) colony or cluster progenitors were initially detected (R.C. and L.H.). In these 2 experiments such progenitors were maintained in both the non-adherent and adherent fractions for at least 8 weeks and there was no tendency for them to decrease with time in culture (Tables XIV and XV). In the other 3 experiments (R.B., J.O., E.T.), progenitors of abnormal (blast) colonies or clusters were not detectable at any time (Tables XIII,XIV, XV).

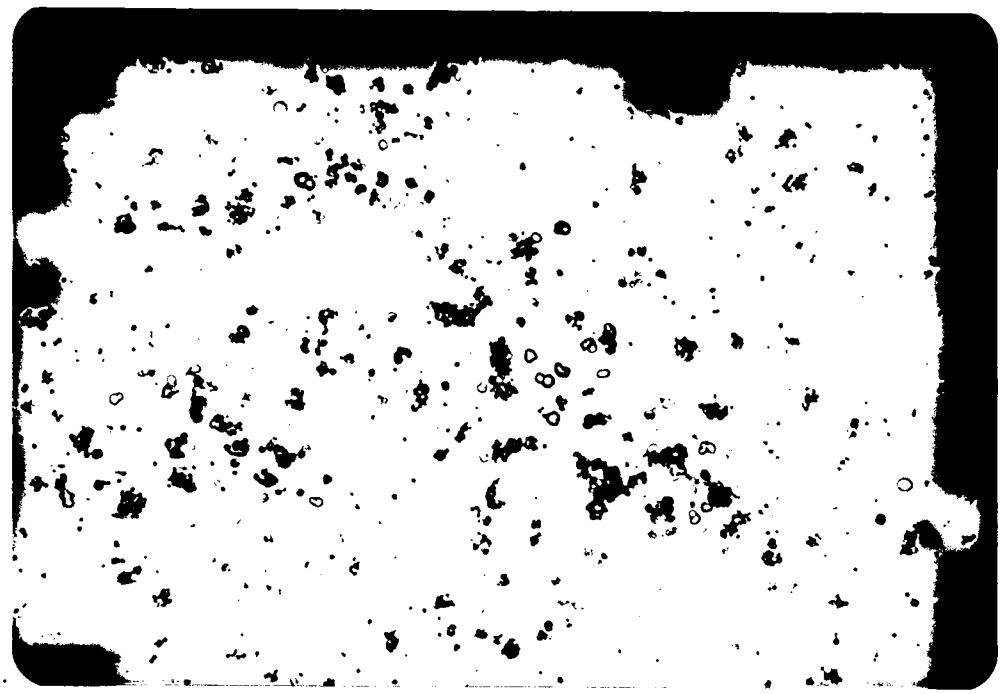


FIGURE 23. Multiple small clusters of abnormal morphology in the methylcellulose assay of 2 week-old non-adherent fraction (culture from patient J.R.) (x80)

TABLE XIII

Results of Methylcellulose Assays of the Initial Marrow Sample of the 13 AML Patients included in this study

Patient		No. of progenitors per 2 x 10^5 buffy-coat cells			
		CFU-E	BFU-E	CFU-C	Abnormal (blast) colonies and clusters
J.B.		122	195	None detected	+
T.E.		13	4	"	+
J.R.		0	2	"	+++
A.McN.		157	11	"	+
P.B.		0	0	"	+
H.M.		32	52	"	+
H.B.		2	0	1	-
K.M.		30.5	10.5	3	-
R.B.		3	10	0	-
R.C.		285	23	None detected	++
L.H.		8	0	"	++
J.O.		0.5	0.5	0	-
E.T.		1	0.5	0	-
Normal° Values (n=11)	Mean	194	92	73	-
	±2SD	66-568	30-284	12-412	-

°From reference 24.

TABLE XIV

Results of Methylcellulose Assays of the Non-Adherent Fraction of Long-Term Marrow Cultures from the 13 AML Patients of this Study

Patient	Abnormal (blast) colonies and clusters		No. of progenitors of normal granulocyte colonies (CFU-C) per long-term culture	
	1-3 wks	4-8 wks	1-3 wks	4-8 wks
J.B.	+	-	None detected	0-101
T.E.	+	-	"	17-65
J.R.	+++	-	"	13-32
A.McN.	+	-	"	20-197
P.B.	+	-	"	1.5
H.M.	-	-	160-570	18-131
H.B.	++	-	None detected	91-120
K.M.	+	-	"	4-180
R.B.	-	-	277	20
R.C.	++	++	None detected	None detected
L.H.	+	+	"	"
J.O.	-	-	"	"
E.T.	-	-	"	"
Controls[o]	-	-	225-21,390	4-1,496

[o]Range of values obtained from concurrently carried long-term normal marrow cultures (see chapter II).

TABLE XV

Number of Hematopoietic Progenitors in the Adherent and Non-adherent Fractions of Long-term Marrow Cultures from the 13 AML Patients Included in this Study

Weeks of Culture	Patient	No. of progenitors per culture							
		BFU-E		CFU-C		CFU-G/E		Abnormal (blast) colonies and clusters	
		A	NA[o]	A	NA	A	NA	A	NA
4	T.E.	15	7	62	49	<7	<3[++]	+	+
	J.R.	86	80	270	38	10	<40	+	+
	A.McN.	14	4	34	60	<1	<1	-	-
	P.B.	<5	<1	5	2	<5	<1	-	-
	R.B.	13	5	87	20	<6	<1	-	-
	L.H.	<7	<10	<7	<1	<7	<10	+++	+
	J.O.	16	3	30	<3	<8	<3	-	-
	Controls*	340	79	1142	682	4	2		
6	T.E.	18	ND[+]	77	ND	5	ND	-	ND
	J.R.	15	2	78	23	2	2	-	-
	A.McN.	26	1	244	197	2	<1	-	-
	H.B.	23	4	364	116	<6	<2	-	-
	L.H.	<7	<4	<7	<4	<7	<4	++	+
	E.T.	<11	<17	<11	<17	<11	<17	-	-
	Controls	79	2	324	93	3	<2		
8 & 9	J.B.	6	ND	6	ND	<3	ND	-	ND
	T.E.	15	ND	38	ND	<7	<3	-	ND
	J.R.	5	ND	5	ND	<3	ND	-	ND
	L.H.	<6	<4	<6	<4	<6	<4	++	+
	Controls	50	3	162	58	<12	<2		

[o] A = Adherent; NA = Non-adherent

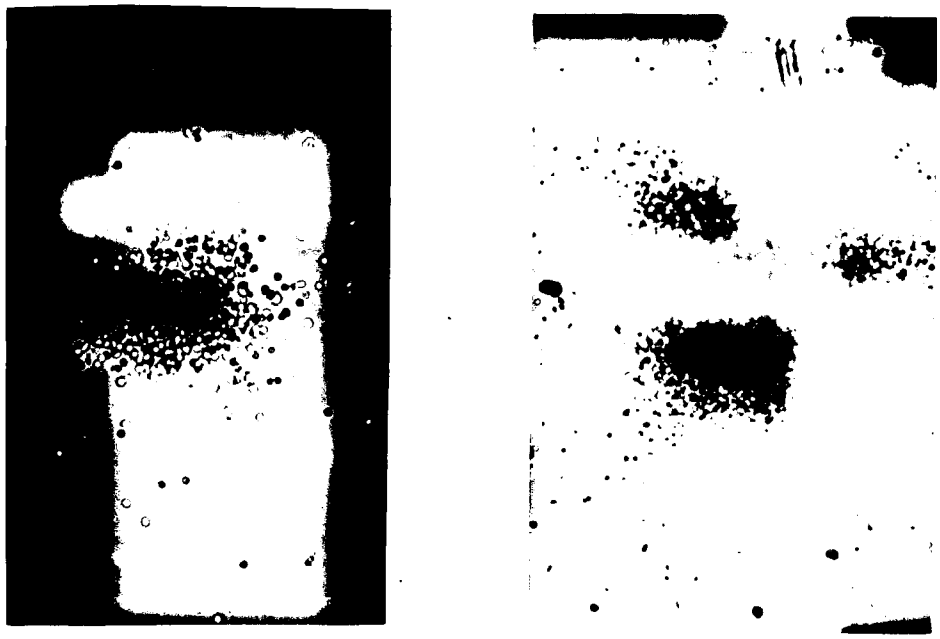
*Values from 31 long-term normal marrow cultures maintained under similar conditions (see chapter II)

[+] Not done (very few non-adherent cells).

[++] None detected; value shown represents the number that would correspond to the detection of 1 colony in the portion of the culture actually assayed.

Behavior of Progenitors of Morphologically Normal Erythroid and Granulocytic Colonies in Long-term AML Marrow Cultures. Normal granulocyte/macrophage colonies were rarely seen in initial assays. CFU-E and BFU-E, however, were detectable in the marrow of most patients studied but usually, although not always, at reduced levels. Those patients whose marrow showed a normal concentration of erythropoietic progenitors were noted to be among those whose WBC counts were not elevated and whose numbers of circulating blasts were relatively low (Table XIII).

In the first 9 experiments, (patients J.B through R.B.), "normal" CFU-C (yielding typical granulopoietic colonies - see Figure 24A and 24B) were readily detectable in assays of both non-adherent and adherent fractions of long-term cultures that had been maintained for 4 weeks or more. The preferential location within the adherent layer of progenitors giving rise to morphologically normal colonies was a constant finding in this study, as it was for long-term cultures established with normal and CML marrow cells (see chapters II and III). Adherent layer assays from 7 of the 9 experiments in which non-adherent CFU-C became readily detectable after 3 weeks also revealed the presence of typical normal marrow adherent layer CFU-C capable of generating very large granulocyte colonies containing at least 500 cells (Figure 24B). In fact, almost as soon as normal progenitors became detectable in long-term AML marrow cultures, they appeared as the predominant type of progenitor present (Tables XIV and XV). In 2 experiments (H.M. and R.B.), "normal" CFU-C were already detectable after only 1 week of long-term culture. Thus all experiments where progenitors of abnormal (blast) colonies or clusters disappeared with time in culture (i.e., the first eight patients) also showed concomitant emergence of "normal" CFU-C. However this latter phenomenon was not limited to such cultures, as exemplified in patient R.B.'s



(A) (B)

FIGURE 24. Morphologically normal colonies observed in colony-assays from long-term AML marrow cultures:
(A) small granulocytic colony in the methylcellulose assay of the 4 week-old non-adherent fraction of cultures from J.R. (x80)
(B) large granulocytic colony observed in methylcellulose assay of the adherent layer of cultures from A.McN. (x80)

cultures. In this case, although the initial marrow contained 90% M1 type blasts and no detectable progenitors of any kind (i.e., <1 per 4 x 10^5 cells), after 1 week of long-term culture, the non-adherent population of 3 x 10^5 cells (a value which is below the normal range, see above), was found to contain very few blasts and 277 CFU-C. This number of CFU-C is just within the lower limit measured for a large number of 1-week-old normal long-term marrow cultures (see chapter II). For 6 of the other 8 long-term AML cultures in which CFU-C were detected after week 4, values also fell within the normal range, although, as in experiment 9, usually close to the lower limit (Table XIV). These kinetics are shown in greater detail in the example shown in Figure 25. In the long-term marrow cultures of this patient, (T.E.), the number of progenitors of normal granulocyte colonies detected in the non-adherent fraction was below the normal range at week 5 but recovered to values in the normal range thereafter. A similar pattern was seen in 3 of the 8 other patients where normal CFU-C became detectable. Even in normal cultures non-adherent CFU-C rarely increase with time. It therefore seems more likely that the apparent increase in CFU-C numbers seen in some AML cultures was more a reflection of increased plating efficiency due to the disappearance of abnormal cells.

CFU-E were rarely detected in either the non-adherent or adherent fraction of long-term AML cultures that had been maintained for 4 weeks or more (data not shown). This is characteristic of the "block" in erythropoietic differentiation seen in long-term cultures maintained according to the procedures used here. The majority of BFU-E present in AML cultures were found in the adherent layer (Table XV), a finding also characteristic of normal marrow cultures (see chapter II). In 8 of the 9 cultures where normal CFU-C were found, BFU-E were readily detectable and in 4 cases CFU-G/E were

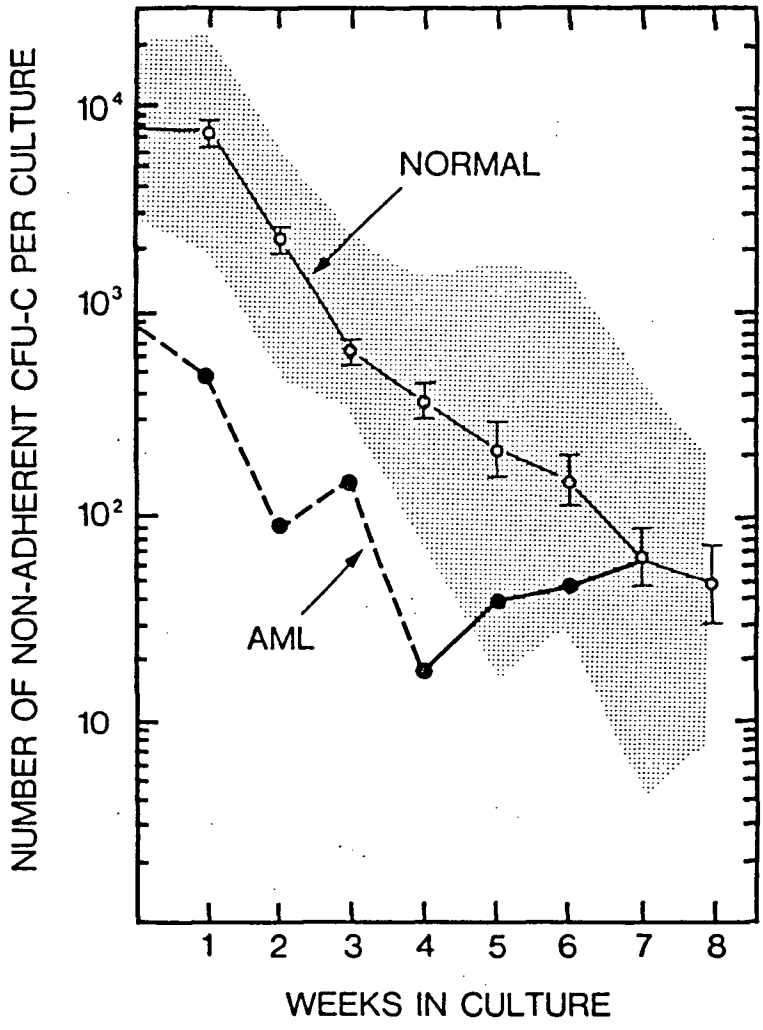


FIGURE 25. Number of CFU-C in the non-adherent fraction of the long-term marrow culture from patient H.B. Dotted line: Estimated number of CFU-C in assay dishes where abnormal colonies were prevalent; solid line: Number of CFU-C in assays where abnormal colonies were no longer detected; shaded area: Range (and mean ± SEM) of CFU-C values measured in normal long-term marrow cultures established at the same time

also detected. The numbers of primitive progenitor types (CFU-G/E, BFU-E and CFU-C) per culture were low compared to normal cultures, but higher than expected if related to calculated input values. For example, in the cultures from patient H.B., assays of 6-week-old long-term cultures revealed the presence of 27 BFU-E, although none had been detected in assays of the initial marrow. Thus the number present after 4 weeks appears to correspond to at least one third of the maximum number of BFU-E that might be assumed to have been present initially. Similarly, in the cultures established from patient J.R., initial assays suggested that long-term cultures were seeded with 250 BFU-E, although after 4 weeks the number detected was 166, i.e., more than two thirds of the apparent input value. Taken together these data suggest that direct assessment of the normal progenitor content of the initial marrow of many AML patients may significantly underestimate the true value due to inhibition of normal differentiation in the presence of sufficient numbers of leukemic blasts.

In patients R.C. and L.H., cells capable of abnormal (blast) colony and cluster formation persisted in methylcellulose assays of both adherent and non-adherent fractions, and no CFU-C were detected at any time, although an occasional BFU-E was found in experiment R.C.'s cultures. Both patients were females over 70 years old. Both failed to achieve remission.

Long-term marrow cultures established with marrow cells from patients J.O. and E.T. demonstrated yet a different pattern. Non-adherent cell assays consistently failed to yield colonies or clusters of any kind as did assays from the starting marrow (Table XIII and XIV). A few BFU-E and CFU-C were detected in the week 4 adherent layer of cultures from J.O. None were detected in the adherent layer of cultures from E.T. (Table XV). Nevertheless, in spite of the absence of progenitors capable of proliferation in

methylcellulose assays, a very large non-adherent cell population (>2 x 10^6 cells) was maintained in E.T.'s cultures for 6 weeks (Figure 26). As shown in Table XII, this included a significant blast component and some leukemic cells that were able to undergo varying degrees of granulopoietic differentiation in culture. The adherent layer in this experiment was of average cellularity at week 6 (2.3 x 10^6 cells). Both patients J.O. and E.T. failed to enter remission.

(D) Cytogenetic Studies

Cytogenetic studies were undertaken in two experiments (J.R. and A.McN.). As shown in Table XVI, all direct marrow metaphases obtained from patient 3 (J.R.) were found to belong to a 9q- population and proliferating normal cells were not detected. For patient A.McN., half of the direct marrow metaphases showed a complex karyotype, the remainder being chromosomally normal. Morphologically normal colonies generated in assays of 4- to 6-week-old adherent layers from both experiments were plucked and analyzed individually. All 34 analyzable colonies proved to be chromosomally normal (Table XVI).

4) DISCUSSION

In the last decade, technical advances in a number of areas have been applied to the study of AML and have rejected the classical concept of this disorder being unique in its pathogenesis with only minor cytological and clinical variations. Analysis of the proliferative (2-4) and differentiative (1,14) potential of leukemic progenitor cells from patients with a clinical diagnosis of AML suggests a marked degree of heterogeneity between patients. This is further strengthened by additional evidence of heterogeneity with respect to the cell of origin (1), the specific chromosomal changes observed

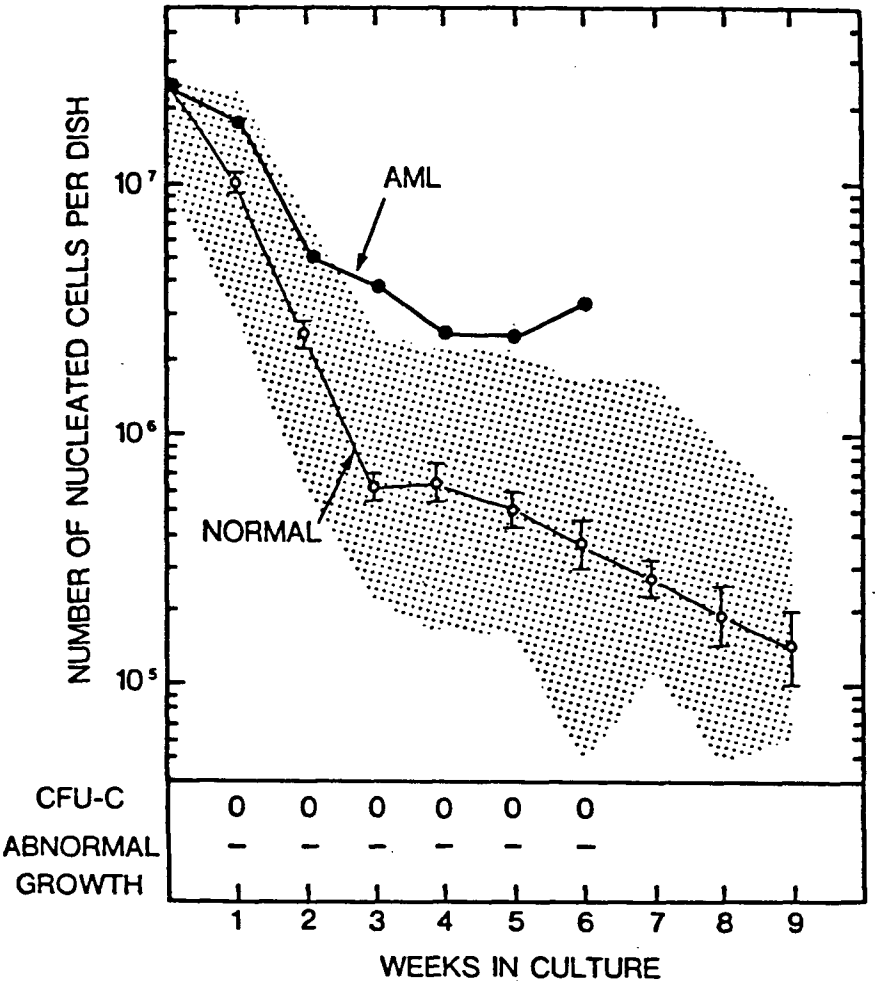


FIGURE 26. Total nucleated cell content of the non-adherent fraction of the long-term marrow culture from AML patient E.T., where no progenitors of any kind were detected in methylcellulose assays. Shaded area shows the range (and mean ± SEM) of values measured in normal long-term marrow cultures established at the same time

TABLE XVI

Comparison of Cytogenetic Findings on Progenitors Maintained in Long-Term AML Cultures from 2 Patients with Initial Results from Direct Marrow Preparations

Patient	Karyotype	Direct Marrow Metaphases	Progenitors in 4-6 wk Adherent Layers		
			BFU-E	CFU-C	CFU-G/E
J.R.	46,XY,9q-	11	0	0	0
	46,XY	0	7	10	2
	Total	11	7	10	2
A.McN.	43,XY,-8,-13,-14,-15,-16,-21,+M_1,+M_2,+M_3 der(16)t(16q;?)	10	0	0	0
	46,XY	10	8	7	0
	Total	20	8	7	0

(15,16) and the possible involvement of particular oncogenes in leukemic cells from different individuals (17,18).

Thus far, however, such studies have been limited either to fresh marrow (or blood) samples or to autonomous cell lines derived from such samples. This was due mainly to the lack of a suitable culture system where stem cell functions could be maintained and analyzed. As these cells are the target cells of most of the human leukemogenic processes, the maintenance of their normal counterparts in vitro was a prerequisiste for studying mechanisms of leukemic cell persistence in vitro. It was shown in the previous chapters that normal primitive progenitors could be successfully maintained for 2 months in long-term cultures established with marrow cells from normal individuals or patients with Ph^1-positive CML.

In the present study we used marrow aspirates from newly diagnosed AML patients to initiate long-term cultures. The kinetics of clonogenic progenitor cells detectable in such cultures was than followed over the next 8 to 9 weeks. Heterogeneity in the behaviour of all types of progenitors was the most striking finding when these cultures were compared one with another. However, from these studies, 3 basic patterns appeared (see in Table XVII). The first was characterized by the detection of significant numbers of BFU-E and CFU-C in long-term marrow cultures maintained for more than 3 or 4 weeks, even though typically none or very few of these progenitor types could be identified in initial marrow assays. Long-term marrow cultures from 9 patients showed this pattern (group 1). In 8 of these, progenitors of abnormal (blast) colonies or clusters were detected early on (after 0 or 1 week in long-term culture), but their numbers declined rapidly and these could no longer be detected after 4 weeks. In the second and third groups (4 patients), CFU-C and BFU-E were rarely detected at any time. In 2, (group 2),

TABLE XVII

Pattern of Growth in Methylcellulose Assays of Long-Term Marrow Culture Cells (Adherent and Non-Adherent) from 13 AML Patients

Group	No. of Patients	Abnormal Growth <3 wks	Abnormal Growth >3 wks	Morphologically/Cytogenetically Normal Colonies
1	9	+ or -	-	+
2	2	+	+	-
3	2	-	-	-

progenitors of abnormal (blast) colonies or clusters were detected both initially and subsequently for the entire duration of the cultures. In the other 2, (group 3), such progenitors were not detected at any time. However, in one of these experiments the maintenance of a leukemic progenitor population might be inferred from the fact that a large population of non-adherent blasts was maintained for at least 6 weeks in spite of the weekly removal of half of their numbers (Figure 26).

These findings raise a number of interesting practical and theoretical issues. First, what is the mechanism that leads to the apparent emergence of progenitors capable of normal differentiation (in excess of that predicted from input values) and the concomitant disappearance of abnormal (blast) colony-progenitors with time in long-term culture? One possibility is that the production of CFU-C (and sometimes BFU-E) from normal stem cells and their subsequent differentiation may be suppressed by the presence of a large leukemic blast population. For reasons not yet clear, it appears that long-term cultures are not usually able to support the maintenance of clonogenic blast progenitors. Accordingly, as these decline in long-term cultures, the mechanism for suppression of normal hemopoiesis would be lost and the progeny of previously quiescent stem cells might become detectable. The increase to near-normal values of the CFU-C population (see Figure 25) might suggest that the size of the residual normal population remains close to normal in AML when first diagnosed. Suppression of normal hemopoiesis by leukemic cells has been suggested by others (19), although these findings remain controversial (20). Nevertheless, it is an attractive explanation for the present findings and is given further support by the finding from long-term CML cultures (see chapter III). In these, the neoplastic Ph^1-positive population also usually declines rapidly often allowing previously undetectable Ph^1-negative progenitors to

become apparent (21). In the present study, a chromosomal marker for the AML population was known for 2 of the patients whose long-term cultures showed emergence of progenitors capable of normal differentiation. In both instances these late appearing progenitors were also found to be chromosomally normal.

A second and related question pertains to the "normalcy" of such progenitors. Although it seems likely that these would have derived from residual stem cells outside of the leukemic clone, this may not have been the case in every experiment. A number of lines of evidence suggest that leukemic transformation may be a multistep process involving a series of genetic changes, the first of which might not necessarily be detectable at the chromosomal level (22). Moreover, the demonstrated ability of clonal AML progeny to execute normal differentiation programs under conditions prevailing in vivo (1,22), as well as following experimental manipulation in vitro (23), suggests that abnormal differentiative behaviour may often be due to secondary changes possibly associated with the evolution of more frankly leukemic subpopulations. Thus neither phenotypic nor cytogenetic normalcy can be taken as rigorous evidence that the progenitors detected in long-term AML cultures were not neoplastic. Because of the typically rapid rate of expansion of the leukemic clone in patients with untreated AML, the most aggressively proliferating subpopulations predominate and earlier stages in their evolution cannot be readily studied. It is therefore not surprising that very little is known about the cellular changes that precede overt disease. The present studies are relevant in this regard since they suggest that long-term culture may offer a novel method for selecting earlier stages of leukemic cell development.

On the other hand, it is already clear from studies of treated AML patients that in many instances hemopoiesis in remission is regenerated from

co-existing nonclonal stem cells (1). In fact, this has stimulated efforts to develop a variety of methods for removing residual leukemic cells from AML marrow samples destined for autologous reinfusion following treatment of subsequent relapses. In view of current technical and practical problems facing investigators in this area, selection in favour of normal elements using the type of culture procedure described here offers an alternative that may be worthy of future consideration.

Finally, a comment is warranted regarding the clinical outcome of patients grouped according to the behaviour of their marrow cells in long-term culture. Obviously, a series of 13 patients is too small to draw any general conclusions. Nevertheless, the fact that all 4 patients whose long-term marrow cultures failed to yield normal progenitors, also failed to achieve a first remission is suggestive that biological parameters of prognostic importance may emerge from more extensive studies of the type described here. Similarly, it will be of interest to determine why some leukemic marrow cell populations appear able to proliferate under conditions set up in long-term culture but not in dilute suspension in methylcellulose, and whether this has clinical significance.

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C H A P T E R V

SUMMARY AND CONCLUSIONS

Considerable evidence indicates that the continuous production of the various mature blood cell types depends on the maintenance of a population of pluripotent progenitor cells. These stem cells, or a subpopulation of them, must therefore be able to respond appropriately to the need for supplying progeny committed to maturation along each differentiation pathway, without exhausting their own numbers. At present, virtually nothing is known about the molecular nature of the mechanisms that mediate this decision to self-renew or differentiate, although evidence exists for both deterministic and stochastic components. The limited rate of progress in this area is due in part to the fact that the behavior of pluripotent cells, and attempts to modulate it, can be assessed only indirectly, by characterization of the differentiated cells produce many divisions later either in vivo or in vitro. As most of the human neoplastic hematopoietic disorders are stem cell diseases, similar limitations have hampered studies of the regulatory anomalies that underly these diseases.

A major step forward was made with the development of a culture system that allowed mouse marrow stem cells to proliferate and maintain their numbers for many months in an environment to which exogenous growth factors were not added. The use of this long-term culture system provided an opportunity to analyse in vitro for the first time not only the mechanisms that regulate murine stem cell behavior, but also the progressive steps of leukemogenesis in that species.

Attempts to adapt this system to the maintenance of human hematopoiesis have been rather disappointing in terms of the duration of a productive culture and the numbers of progenitors recovered, although detailed examination of the progenitor populations maintained in this system as a whole had not been assessed, and in particular, the content of the adherent layer had not been examined.

The first goal of my thesis project was to define the possible role of the adherent layer in the maintenance of hematopoiesis in human long-term cultures initiated with normal bone marrow cells, and to determine whether or not this system could be used to analyze the behavior of neoplastic populations.

Long-term cultures established with normal human marrow cells.

The initial part of this work was mainly technical, aimed at obtaining a system optimized for both the establishment of a functional adherent layer, and the prolonged growth of normal progenitor cells. This meant first optimizing the initiation and feeding procedures so that hematopoiesis could be reproducibly maintained over 7-8 weeks and second developing a non-toxic enzymatic procedure that would release the cells comprising the adherent layer into a suspension suitable for subsequent assessment in methylcellulose.

Forty-one long-term cultures were established with normal human marrow cells and the progenitor cell content of both the adherent and non-adherent fractions was assessed at weekly intervals. Results consistently showed that hematopoietic progenitor cells were unequally partitioned between the 2 fractions. Most of them were part of the adherent layer after 4 weeks. In addition, the more primitive precursor cells (i.e., pluripotent stem cells and high proliferative potential CFU-C) were exclusively located within the adherent layer after 4 weeks.

These results provide strong support for the idea that in these human long-term marrow cultures early hematopoietic events may be regulated by short range interactions between stem cells and stromal elements of the adherent layer. Current evidence suggests that this is a key feature of the long-term marrow culture system as originally characterized in the mouse (1). Additional evidence that hematopoiesis associated with the adherent layer is an active process has been obtained more recently. This study has shown that the primitive progenitor types within the adherent layer are actively cycling (2). However, as yet very little is known about the properties of the non-hematopoietic adherent cell populations that may be involved in these regulatory phenomena, or of the mechanisms by which they might influence hematopoietic decisions. Further analysis of the adherent layer organization and of the molecules involved in cell-cell and cell-substrate connections might provide new clues to these questions. This may be approached by attempts at reconstituting in vitro a "functional" microenvironment (i.e. adherent layer) from well-defined adherent cell populations or their extracellular matrix products.

Long-term cultures established with CML or AML marrow cells.

Clonal dominance is a key feature of both CML and AML. However, the mechanisms that are responsible for this are largely unknown. One major consequence of the amplification of the abnormal clone is that it prevents detection of non-malignant cells and progenitors in fresh samples of blood or marrow even though these are known to be present in most AML and some CML patients at diagnosis. Several mechanisms have been proposed to explain the failure of these normal progenitors to differentiate in vivo. For example, it has been suggested that there may be an inhibitory effect of leukemic cells on normal differentiation pathways. However, few studies have attempted to

analyze the level at which suppression occurs, or how it is mediated. These questions are not readily approached using short term assays, but may be analyzable in a culture system which allows stem cell properties to be maintained.

Therefore, when it proved possible to reproducibly maintain active hematopoiesis for several weeks in normal long-term marrow cultures, it was appropriate to investigate the usefulness of the system for studies of progenitors in CML and AML. In both of these disorders the neoplastic progenitor populations can be easily distinguished from their normal counterparts either by chromosomal markers (AML and CML) or by their relative ability (or inability) to generate normally differentiated end cells (AML).

CML studies. Thirteen long-term cultures experiments were established with marrow cells from 11 untreated Ph^1-positive CML patients. The most striking feature of all these was the rapid decline of all progenitor types in both the adherent and non-adherent fractions. These abnormal kinetics were subsequently clarified when cytogenetic analysis of the progenitor populations, performed in 7 experiments, showed that in all, Ph^1-positive precursors disappeared very rapidly after initiation of the long-term culture and in all except one experiment, this led to the detection of usually small numbers of Ph^1-negative progenitors. There was no case of persisting mosaicism beyond 4 weeks. Since these experiments were completed, an additional 12 patients have been studied. These have confirmed that the rapid disappearance of Ph^1-positive precursors is a general phenomenon in CML patients, including treated ones, and that residual Ph^1-negative progenitors can be detected in most of the patients at diagnosis, even though they are usually initially undetectable. Heterogeneous behavior of the Ph^1-positive populations has also been encountered. In a minority of patients, Ph^1-negative

progenitors are not detected, and in one experiment, Ph^1-positive cells were maintained at much higher levels than usual for at least 6 weeks (3).

The striking differential ability of Ph^1-positive and Ph^1-negative progenitors (whose non-clonal origin has been demonstrated in one case (4)) to become part of the adherent layer of long-term marrow cultures suggests that this selection may be a result of differences in the properties of normal and neoplastic stem cells. As normal (or Ph^1-negative) differentiation processes occuring in the adherent layer are likely to depend on close interactions with stromal elements, this might indicate that these interactions do not operate similarly with Ph^1-positive cells. It will be of great interest to investigate further whether the failure of Ph^1-positive progenitors to be maintained in vitro is due to the lack of an appropriate environment specifically required by the Ph^1-positive cells or to altered properties of the cell itself preventing proper interactions with stromal structures which would otherwise support their proliferation. Modulation of culture conditions in the long-term system is known to profoundly influence the types of proliferating cells and it might be conceivable to achieve altered conditions that might allow the growth of Ph^1-positive progenitors as has been recently suggested (5).

The persistent growth of Ph^1-positive progenitors in long-term marrow cultures of one CML patient clearly indicates that heterogeneity exists in this disorder, in spite of apparent homogeneity in terms of clinical presentation, chromosomal findings, and the differentiation potential of its progenitor cells in short-term assays. Whether or not the different growth patterns displayed by PH^1-positive populations in long-term marrow cultures from different patients define different types of diseases with different neoplastic cells properties and regulation requires further investigation.

AML studies. Heterogeneity in the maintenance of neoplastic progenitors in CML long-term cultures was unexpected, but is perhaps less surprising in AML where wider ranging heterogeneity in a number of clinical and biological parameters has been recognized for some time. In the present studies, long-term cultures were established with marrow cells from 13 newly diagnosed AML patients. Detection of leukemic progenitors was based on their ability to generate colonies of abnormal morphology in methylcellulose assays. Assessment of these, as well as of progenitors capable of generating phenotypically normal colonies revealed three patterns in the 13 experiments performed: (i) Initial evidence of leukemic precursors, which then rapidly disappeared over the next 3 to 4 weeks (9/13 patients); (ii) Initial evidence of leukemic progenitors which then persisted in both the non-adherent and adherent fractions for at least 8 weeks (2/13 patients). (iii) No leukemic progenitors detectable at any time in colony-assays (2/13 patients) from fresh marrow or long-term cultures cells although in one experiment, high blast cell counts persisted in the non-adherent fraction. Progenitors giving rise to morphologically normal granulocyte colonies were detected after 4 weeks exclusively in the first group and then in numbers close to what would have been expected in normal control cultures (both fractions) that had been maintained for an equivalent period.

In 2 patients with initial chromosomal abnormalities, the cytogenetic normalcy of the progenitor cells found in the adherent layer after 4 weeks confirmed that colonies considered as phenotypically normal did not arise from the leukemic precursors initially predominant. Nevertheless, the possibility that the emerging "normal" progenitor cell population might, in at least some cases, have still been part of a preleukemic population is not ruled out. Recent evidence suggests that cytogenetically normal but still neoplastic

cells may be present in some patients with AML and repopulate the hemopoietic system after remission induction (6). Long-term marrow cultures may then offer a system to analyze early stages in the leukemic process, a step not usually accessible to study because the relevant cell populations are masked by the predominance of the more aggressive populations. Until an AML marrow from a constitutional mosaic (e.g., a female G6PD heterozygote) is available for assessment in long-term culture, it will be impossible to determine whether the selection observed to date in favour of cells capable of normal differentiation also distinguishes between cells capable of clonal overgrowth or not.

Failure of non-malignant stem cells to differentiate in vivo is well known, although the cell types involved have not been defined. The present study suggests that, as in CML, suppression may be more pronounced at the terminal stages of differentiation since the detection of a normal population from week 4 to 7 inversely parallels the disappearance of the leukemic population, and exhibits kinetics suggesting their presence, or the presence of their precursors, at earlier times when they could not be detected.

General comments.

The most significant finding of this work was that the capacity for clonal dominance that prevails in vivo in AML and CML is typically reversed in vitro. Interestingly, however, this was not the only growth pattern encountered, and in a few AML and CML experiments, dominance of the neoplastic clone persisted in vitro. Future work will be necessary to clarify why neoplastic progenitors decline in long-term marrow cultures, and to delineate the biological and clinical significance of the heterogeneity described in this work. However, since the disappearance of the leukemic populations occurs at a similarly rapid rate in both AML and CML experiments, it seems likely that the same or related mechanisms, yet to be determined, may be involved.

Long-term cultures represent a complex system, and any disturbance in the maintenance of hematopoiesis may have multiple origins (i.e., involving either stromal cells or stem cells). From the first part of this work it appears that normal primitive hematopoietic progenitors require an intimate association with non-hematopoietic cells in the adherent layer to be maintained for periods of 8 weeks. Failure of such interactions to occur, which may result if neoplastic cells are intrinsically altered in such a way so as to prevent them from establishing close connections with stromal cells, provides one explanation for the non-maintenance of neoplastic stem cells in long-term marrow culture. On the other hand, neoplastic stem cells may also require some essential and specific nutrient (or accessory cell), usually not provided in a culture system optimized for the maintenance of stem cells capable of normal differentiation. It is therefore conceivable that the growth of neoplastic cells could be achieved by some, perhaps minor, alteration of the parameters of this multi-component system. A striking example of such a modulation has recently been described in long-term mouse marrow cultures infected with the src oncogene (7). Such a possibility would be of considerable interest for the further understanding of the neoplastic process and might then lead to mechanistic clues that could be exploited to therapeutic advantage.

The clinical relevance of our findings cannot be determined at this stage, because of the small numbers of patients thus far studied. However, some questions may be asked. For example: (i) Does the persistence (or disappearance) of neoplastic progenitors in long-term marrow culture correlate with prognosis (i.e., probability and/or duration of remission)? (ii) Is there any future interest in long-term marrow cultures as a potential source of normal stem cells for autologous transplantation? (iii) Can leukemic stem cell subpo-

pulations be selected or their differentiative behavior be modulated under defined conditions in vitro? There is little doubt that in the near future, fu ndamental and clinical problems in hematopoiesis will be addressed more and more through the development and use of molecular genetic approaches (8,9). Because they provide, in human as in mouse, the only in vitro system where long-term stem cell self-renewal and differentiation can occur in a regulated fashion, long-term marrow cultures offer a powerful methodology to monitor the modulation by these new technologies (e.g., gene transfer (8,9)) of pluripotent human stem cell properties.

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