STUDIES OF THE ROLE OF MESENCHYMAL CELLS IN THE REGULATION OF HEMOPOIESIS

by

LOUIS A. GABOURY

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ABSTRACT

Hemopoiesis is thought to be regulated in part by specific, but as yet undefined, interactions between primitive hemopoietic cells and fixed, non-hemopoietic marrow elements collectively referred to as the stroma. Recently, a marrow culture system has been described that allows the maintenance of primitive human hemopoietic progenitor cells for many weeks in the absence of exogenously added hemopoietic growth factors. The formation of a heterogeneous adherent layer in which many stromal elements are found appears to be important to the maintenance of hemopoiesis in this system. As part of the overall goal of delineating the cellular and molecular interactions involved, my first objective was to develop an experimental system for assessing the hemopoiesis-sustaining function of the adherent layer of long-term human marrow cultures. This required the identification of a suitable procedure for separating the hemopoietic and non-hemopoietic regulatory components so that the former could be used to quantitate the function of the latter. This was achieved using irradiation to selectively inactivate residual hemopoietic cells in long-term culture adherent layers, and using a medium containing cis-4-hydroxy-L-proline to selectively inactivate stromal cells and their precursors present in suspensions of unseparated human marrow which were then added back in co-culture experiments.

My second objective was to develop a strategy for obtaining purified populations of cells corresponding to the various mesenchymal cell types in long-term adherent layers. I therefore prepared a high titre SV-40 virus stock and used it to establish permanent, cloned lines from human marrow "fibroblast" colonies, long-term culture adherent layers, and umbilical cord endothelial cells. Characterization of the transformants generated showed

that they were all positive for SV-40, and in general expressed the phenotypic characteristics of the cells originally infected. Functional studies showed that these transformants, like their normal counterparts, respond to activation by producing two types of hemopoietic growth factors.

These studies suggest that marrow mesenchymal cells may regulate the growth and maintenance of primitive hemopoietic cells by producing hemopoietic growth factors in response to appropriate perturbation. The availability of permanent cloned lines of human marrow stromal cells should facilitate future analysis of these events at the molecular level.

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CHAPTER I

INTRODUCTION

1) ORGANIZATION OF THE HEMOPOIETIC SYSTEM

(A) Hemopoietic Cells

The hemopoietic system consists of a lymphoid and a myeloid arm, both of which are thought to originate from common ancestor cells that have undergone a series of migrations from their original location in the yolk sac to their final destination in the functional hemopoietic organs of the adult, i.e. the bone marrow, spleen, thymus and lymph nodes (1) (see Figure 1).

All mature myeloid cells (red blood cells, granulocytes, monocytes and platelets) represent non-dividing "end" cells that survive for relatively short periods of time and are, therefore, being continuously replaced throughout adult life (2). The production of new blood cells from more primitive proliferating precursors normally occurs in the bone marrow of the adult (3).

The structure of the hemopoietic system is currently viewed as consisting of four major cell compartments (4). The most primitive are the **hemopoietic stem cells**, cells that have the potential both to self-renew and to differentiate into each of several lineages. Stem cells give rise to an intermediate, transient compartment of **progenitors** of various types that have undergone different degrees of lineage restriction but still have a considerable proliferative potential. Neither pluripotent stem cells nor

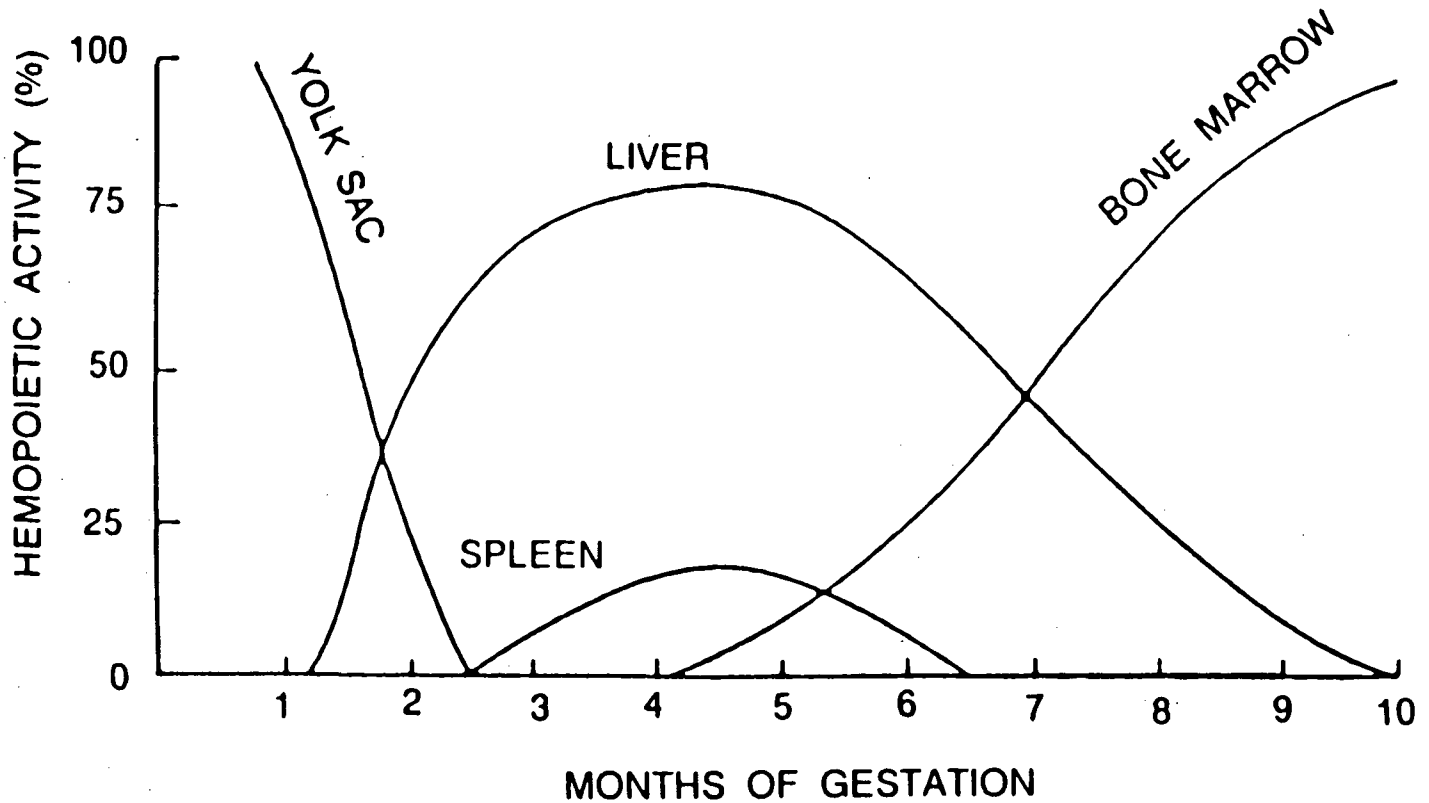

FIGURE 1. Major Sites of Hemopoiesis in the Human Embryo and Fetus.

lineage-restricted progenitors are uniquely distinguishable morphologically, either as a class, or from one another. The progenitors, in turn give rise to a compartment of morphologically recognizable **precursors** that undergo a limited number of divisions, 3-5, in concert with the completion of terminal maturation. The fully differentiated, **non-dividing blood cells** represent the last compartment. Generally assumed in this model, is that differentiative transitions between compartments are unidirectional (cells cannot increase their differentiative potential), and that there is a progressive loss of proliferative potential as cells become restricted in their differentiation potential (4,5).

Assays for Hemopoietic Stem Cells. The first quantitative colony assay for pluripotent stem cells was described for murine cells in 1961 (6). It involves injecting heavily irradiated histocompatible mice with an appropriate number of hemopoietic cells and counting, 1-2 weeks later, the number of macroscopic nodules that have appeared on the surface of the spleen. Cytological and chromosomal marker studies of the cells within such nodules revealed each to be a bonafide clone derived from a single, pluripotent cell (7,8). Injection of cells from primary spleen colonies into lethally irradiated secondary recipients was found to result in the formation of new spleen colonies, again of multi-lineage composition (9). This was the first formal demonstration of the existence in adult hemopoietic tissue of a cell that can self-renew, and that also has the potential to undergo a large number of differentiative divisions along several lineages. Further studies pointed out that the first appearing spleen colonies (seen within 7-9 days after transplantation) are derived from cells that are neither multipotential nor self-maintaining (10) in contrast to the spleen colonies visible at later

times (12-14 days after transplantation). There is now much evidence to suggest some overlap between the progenitors of the late appearing spleen colonies and cells capable of long-term repopulation in mice (11). In addition, recent experiments with retrovirally marked mouse marrow cells have confirmed that single cells can repopulate the entire lymphoid and myeloid system of both primary and secondary recipients (12,13).

Obviously, a comparable in vivo assay for a pluripotent hemopoietic stem cell cannot be performed in humans. However, several lines of evidence indicate the existence of hemopoietic stem cell populations analogous to those identified in the murine system. First, in chronic myelogenous leukemia (CML), detection of a specific chromosomal marker, the Philadelphia chromosome (Ph^1) in all myeloid cells and occasionally in B-lymphoid cells, but never in bone marrow fibroblasts, has supported the notion of a pluripotent hemopoietic stem cell in man that is the target of neoplastic transformation in CML (14,15). Second, studies of a number of women heterozygous for the X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD), who also had one of various myeloproliferative disorders (including CML) have also traced back the origin of the expanded neoplastic clone in each case to a single transformed pluripotent hemopoietic stem cell (16,17,18). More recently, probes for polymorphic regions in the X-chromosome, that are differentially methylated after X-chromosome inactivation in females, have been used to confirm the origin of human myeloproliferative disease clones in pluripotent hemopoietic cells (19). This latter approach has now also been used to assess the clonality of populations in human recipients of normal, allogeneic marrow transplants. In at least one case to date, long-term monoclonal hemopoiesis of donor origin has been documented (20). Thus, it seems very likely that the

conceptual framework of hemopoietic cell differentiation developed from murine studies will apply to the human sytem.

Assays for Hemopoietic Progenitors. Hemopoietic progenitor cells are present at very low frequencies (10^{-3} to 10^{-6}) in normal hemopoietic tissue and have no distinctive morphological features. However, such cells can be identified indirectly by their ability to proliferate and differentiate in vitro. Hemopoietic colony assays involve suspending the cells to be tested in a semi-solid culture medium containing appropriate nutrients, serum (or the essential components contained in serum) and a source of hemopoietic growth factors, either in crude preparations or as highly purified (natural or recombinant) molecules. Depending on the nature and concentrations of the growth regulatory molecules present in the cultures, single, double, or multi-lineage colonies of daughter cells can be obtained (21,22,23). Progenitors are defined by the types of colonies they produce, both in terms of the size of the colony, and its time of maturation and ultimate composition. These parameters appear to be linked and invariant under most circumstances and therefore provide reproducible indicators of the proliferative and differentiative potential of different types of progenitors (24,25). Progenitors categorized in this way show differences in surface antigen expression (26), and may differ in their responses to cell cycle specific agents (27) and to various growth regulatory stimulators (28). Such findings have helped to validate the assignment of the various types of progenitors to a specific location and a relative rank order in the hemopoietic hierarchy (see Figure 2). Cells that form colonies consisting exclusively of granulocytes, monocytes, or both, are named respectively: colony-forming unit-granulocyte (CFU-G), colony-forming unit-monocyte (CFU-M), and colony-forming

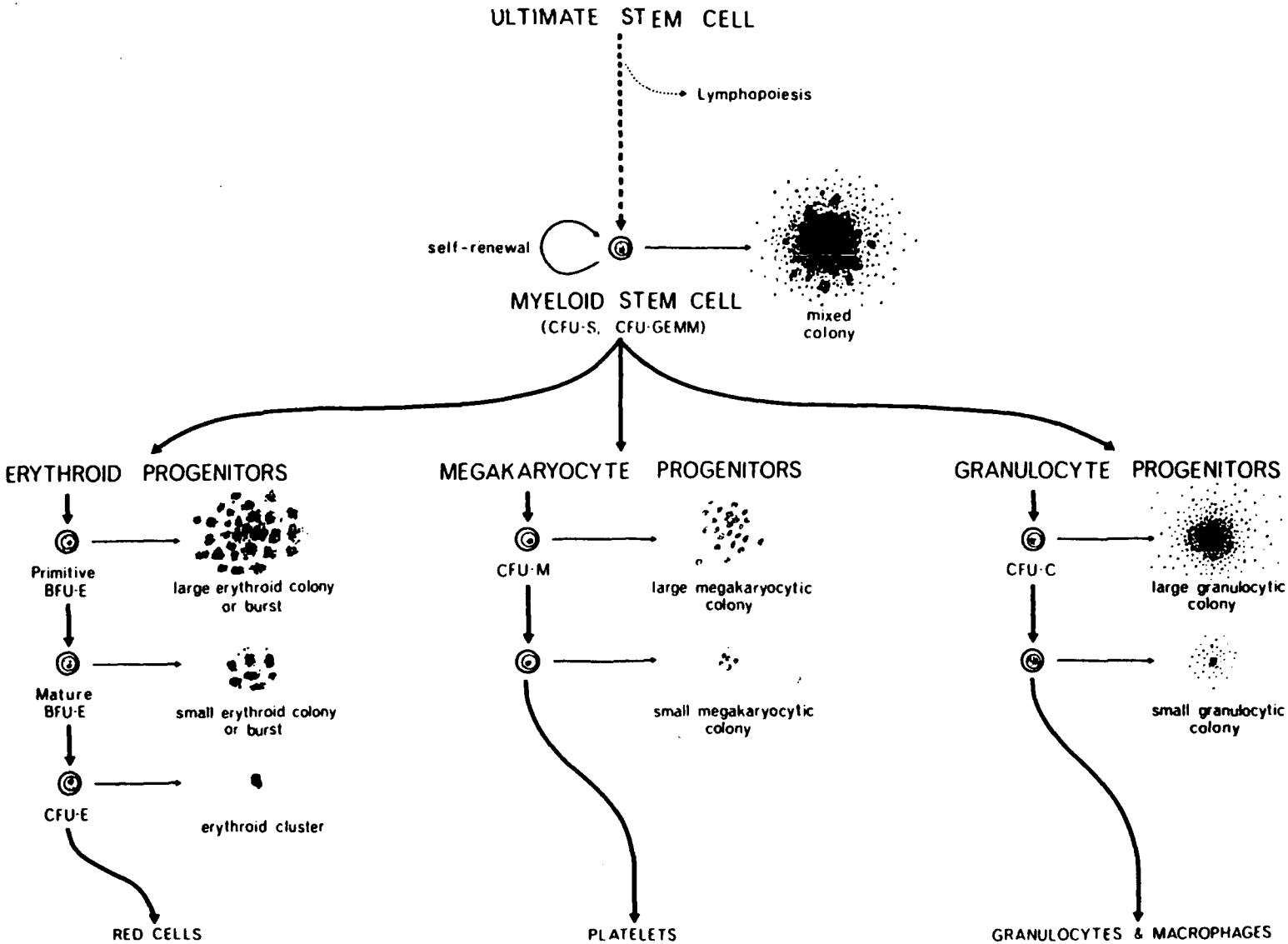

FIGURE 2. Schematic Representation of the Hemopoietic System as Defined by Clonogenic Assay for Pluripotent and Committed Progenitors.

unit-granulocyte/monocyte (CFU-GM). Erythroid lineage-restricted progenitors have been subdivided into categories referred to as colony-forming unit-erythroid (CFU-E), and burst-forming unit-erythroid (BFU-E), according to the number of hemoglobinized cell clusters they generate, the latter often being further subdivided into primitive and mature BFU-E subclasses (21). The same principle has led to the naming of progenitors of colonies of megakaryocytes (CFU-Mk), and of mixed colonies containing granulocytes, erythroid cells, megakaryocytes and monocytes (CFU-GEMM).

Although it is clear that CFU-GEMM represent a primitive pluripotent cell type, those detected in normal marrow exhibit only a limited capacity for self-renewal in vitro. However, very recently a type of CFU-GEMM that shows delayed initiation of proliferation in vitro leading to the production of small "blast colonies" when most other colonies have already matured, has been described. These blast colonies eventually will go on to form pure or mixed colonies, but by replating can be shown to consist of CFU-GEMM, (and CFU-S in the mouse) as well as other more restricted progenitor types (29). Work is now ongoing in many laboratories to analyze the relationship between the progenitors of these blast colonies (referred to as S-cells or CFU-blast) and cells capable of long-term repopulation in vivo.

In summary, clonogenic assays have been developed that allow most classes of myeloid progenitors in both murine and human marrow to be detected, although there is still controversy about the exact relationship between stem cells and progenitors of multi-lineage and "blast" colonies.

(B) Stromal Cells of the Bone Marrow

In addition to precursors of cells that are destined to circulate in the blood, the bone marrow also comprises a variety of cells collectively termed the marrow stroma (Figure 3A and 3B). Ultrastructurally, several stromal cell types have been identified including endothelial cells, fibroblasts/adventitial reticular cells, fat-accumulating cells or adipocytes, osteoblasts, and osteoclasts (30).

Macrophages. Because of their putative hemopoietic supportive functions, fixed marrow macrophages have long been considered part of the stroma. However, marrow macrophages are derived from hemopoietic stem cells and not from the mesenchyma like all other marrow stromal cells (31,32). Marrow macrophages are engaged in active phagocytosis where they play a role in the disposal of particulate matter and cell debris. Numerous hydrolytic enzymes detectable by conventional cytochemical methods are present in their cytoplasm in association with membrane-bound lysosomes (33). Macrophages are also associated with terminally differentiating erythroid cells in the marrow and in this situation are believed to play an important role in the delivery of iron to the hemoglobin-producing erythroblasts. It has also been suggested that marrow macrophages in these erythroblastic islets may be involved in nucleophagocytosis at the time when the erythroblast extrudes its nucleus to develop into a reticulocyte. Consistent with their hemopoietic origin, macrophages also express the hemopoietic markers T200 and the specific monocytic markers LeuM1 and LeuM3, but are not involved in collagen synthesis (34).

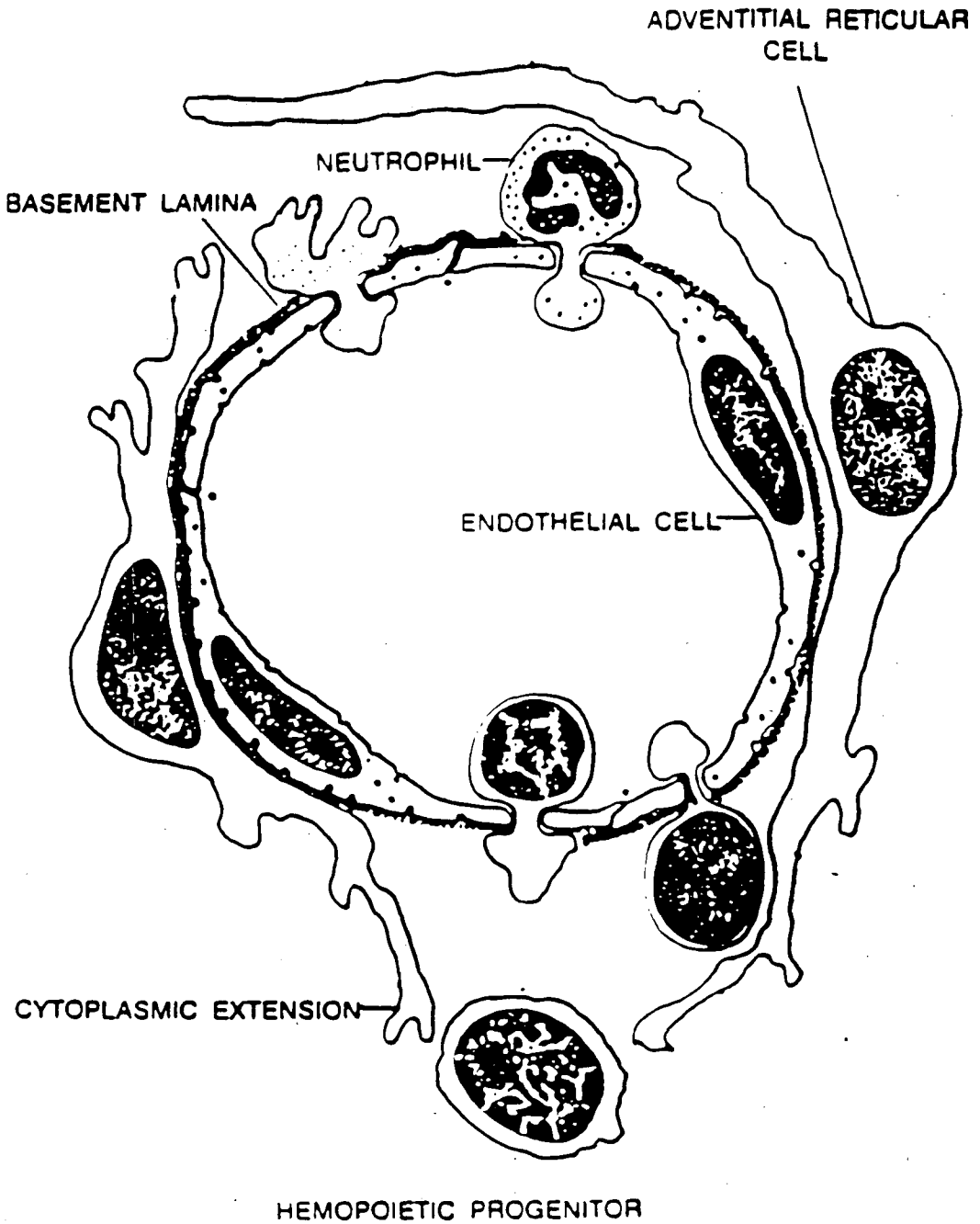

FIGURE 3. Schematic Representation of Bone Marrow Histology.

A) Cellular Arrangement Around a Marrow Sinus. The wall is lined by endothelial cells which are separated from adventitial cells by a discontinuous basement membrane. Hemopoietic cells are present in the extra-vascular spaces.

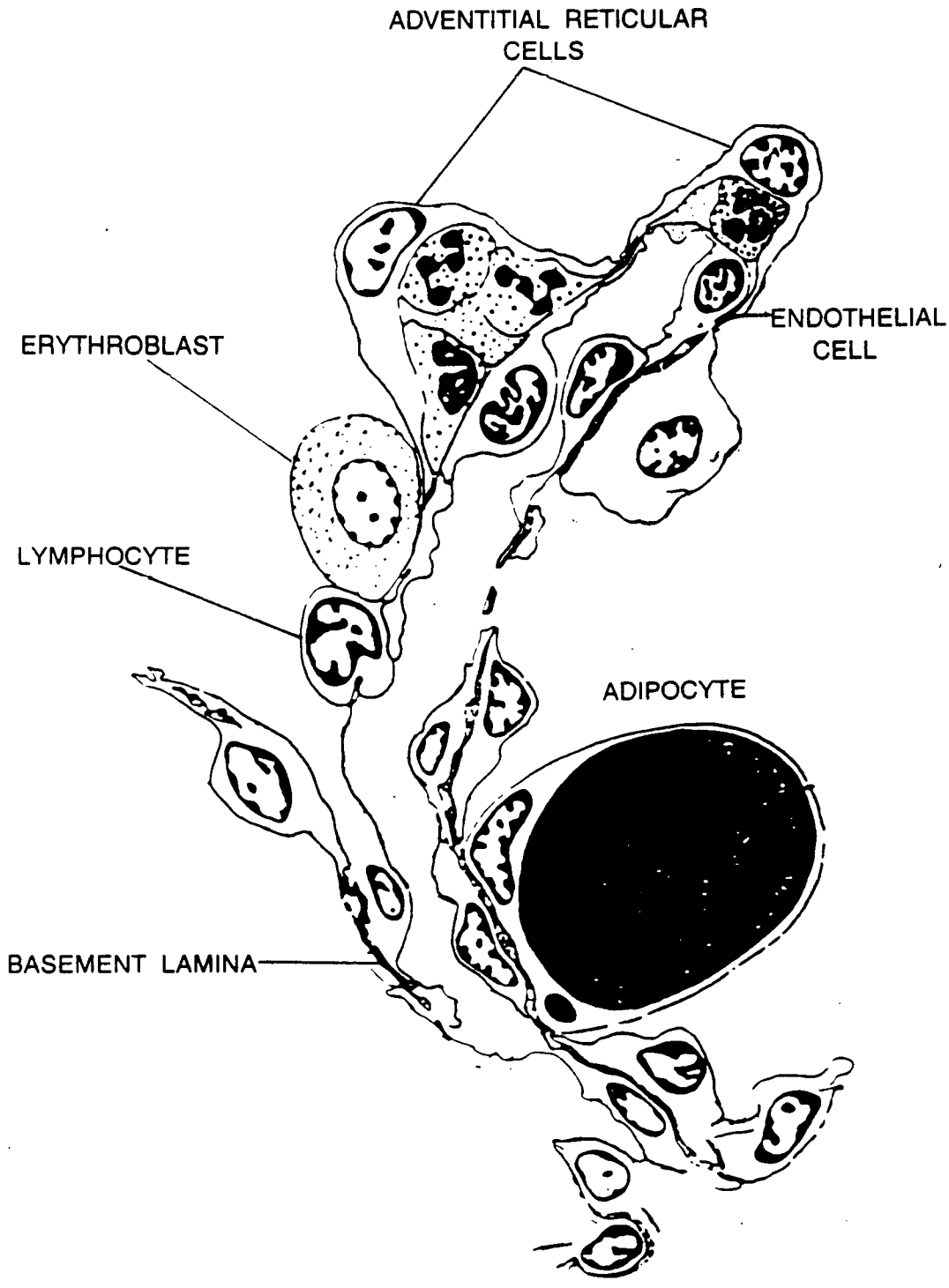

FIGURE 3. Schematic Representation of Bone Marrow Histology.

B) Longitudinal Section of a Marrow Sinus. An adventitial cell which has accumulated fat is shown on the right.

Endothelial Cells. These cells provide the internal lining of the marrow sinusoids and are actively involved in regulating the migration of newly formed blood cells out of the bone marrow. In contrast to endothelial cells located elsewhere in the body, endothelial cells in the bone marrow are attached by loose overlapping junctions (35). They are supported by a thin basement membrane made up of a number of extracellular matrix proteins including laminin, fibronectin, and collagen type IV. In addition, they actively synthesize a number of molecules, one of which, the Factor VIII-related antigen, serves to distinguish endothelial cells from other mesenchymal cell types (36).

Fibroblasts/Adventitial Reticular Cells (ARC). Adventitial reticular cells form a discontinuous cell layer on the abluminal surface of marrow sinusoids. They have an abundant cytoplasm and numerous cell processes and are involved in the production of a supportive meshwork of collagen fibers and reticulin fibers (an argentiphillic mixture of collagen fibers and proteoglycans) (37). The exact relationship of fibroblasts to ARC is still debated but fibroblasts likely represent ARC actively involved in the secretion of collagens (mostly types I and III).

Fat-Storing Cells/Adipocytes. The precise origin of these cells is also somewhat controversial but they are currently believed to be closely related to ARC (38). They are not thought to be lipid-laden macrophages as they fail to express any monocytic markers (39). They also differ from adipocytes characteristic of other tissues since they accumulate lipids in the presence of hydrocortisone but not in the presence of insulin (40).

Osteoblasts. Osteoblasts in the marrow are sometimes observed to be in close association with cancellous bone trabeculae. Osteoblasts are probably derived from cells categorized morphologically as preosteoblasts. Preosteoblasts are fibroblastic cells capable of proliferation located near osteoblasts. They are thought to play a role in the regeneration of the marrow stroma after depletion of the marrow cavity (41).

Osteoclasts. Osteoclasts and their precursors are present near bone surfaces and within cavities in bones. There is now a large body of evidence to indicate that osteoclasts are derived from hemopoietic stem cells via blood-borne mononuclear cells (41). Studies with quail-chick chimeras in which quail cells can be recognized by their specific chromatin organization (32) have confirmed previous data suggesting that osteoclasts, monocytes and macrophages arise from a common ancestor cell. More recently, studies with beige mice (42) that have giant lysosomes in their granulocytes, monocytes and osteoclasts but not in their fibroblasts or in osteoblasts provided further evidence that osteoclasts and monocytes have a common origin.

(C) Stromal Cell Products

As mentioned, marrow stromal cells participate in the formation of a complex extracellular meshwork of fibrous and non-fibrous proteins. Various molecules are known to be present. Some of these have had their genes cloned and their amino acid sequences deciphered. Others have been purified to homogeneity, while some are still ill-characterized but may nonetheless be crucial to hemopoiesis.

Collagens. Collagen is the single most abundant protein species in the marrow stroma. It is secreted by a number of mesenchymal cells (ARC/marrow fibroblasts, endothelial cells) and laid down extracellularly (Figure 4). The collagen molecule has 3 important features: 1) It consists of 3 intertwined α chains forming a stable triple helical structure. 2) Every third residue in the α chain is a glycine. 3) The content of proline and its hydroxylated form is characteristically high. Collagens are further classified into different types according to the nature of their α chains. In the marrow, collagen type I predominates but collagens type III and IV are also present. There are indications that the primary structure of the α chains determines the physical and chemical properties of the mature collagen molecule. This is best exemplified by the important differences which exist between collagen I and IV. Whereas collagen I self-assembles into fibrous proteins, collagen IV has no such propensity.

Collagen biosynthetic pathways are now well characterized. The first step consists of the formation of individual α chains in the rough endoplasmic reticulum. α chains are next hydroxylated at proline and lysine residues, glycosylated at selected hydroxylysine sites, and converted into a stable triple helix. The procollagen molecule is then exported to the interstitium and trimmed of its non-helical amino and carboxy extensions by specific peptidases. Lastly, cross-linking of collagen I and III, catalyzed by the enzyme lysyl oxydase helps to convert single collagen molecules into tight bundles of collagen fibers. Reticulin fibers, putative ARC products, consist of a collagen type III core coated with proteoglycans. The fibers are characteristically present in the interstitial tissue of the marrow where they form a typical arborizing network upon impregnation with a reducible silver salt. Recently, using the matrix induced marrow model, evidence has been

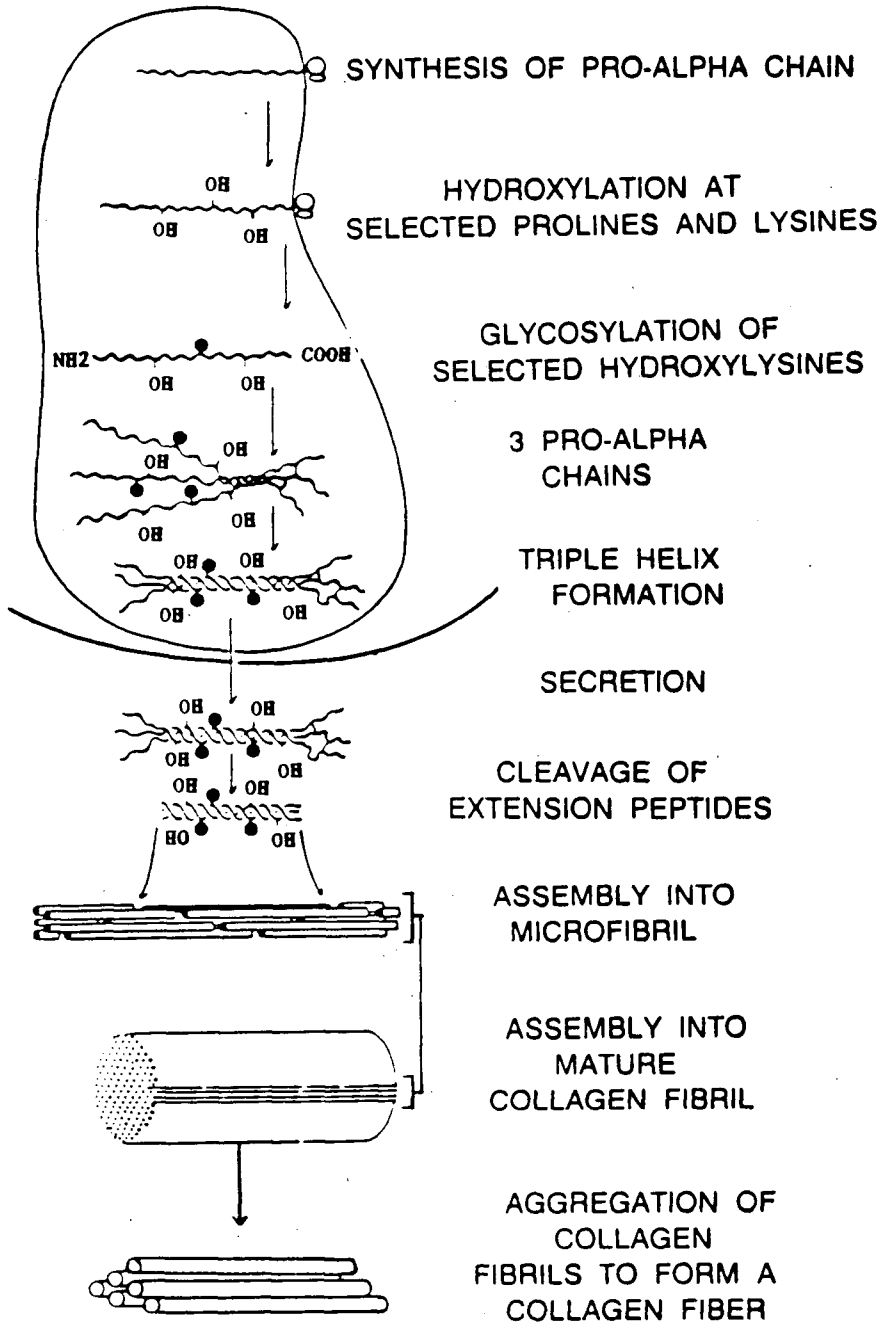

FIGURE 4. Diagrammatic Representation of the Various Intracellular and Extracellular Molecular Events Involved in the Formation of a Collagen Molecule.

presented that collagen III may actually be surrounding nests of hemopoietic cells (43). The significance of these findings is not clear at present. Collagen type IV is the major component of basement membranes. In the marrow, it forms a fine woven meshwork in close association with endothelial cells. The presence of a number of sugar residues have been proposed to explain the conservation of its non-helical extensions extracellularly and thereby the lack of self-assembly into fibrous forms (44).

Laminin. Laminin is an extremely large glycoprotein (900,000 daltons), widely distributed amongst the various basement membranes (45). It has a peculiar crucifix-shaped configuration that allows specific portions of the molecule, also known as domains, to interact specifically with adjacent cells and the extracellular matrix components proteoglycans and collagen IV. This molecule is secreted by various epithelial cells, endothelial cells and some smooth muscle cells (45). In addition to its binding ability, laminin is thought to play a role in regulating the types of macromolecules that pass across the basement membrane (46,47).

Fibronectin. Fibronectin is also a major mesenchymal cell product (48). It has a high molecular weight (200,000-250,000 daltons) and is normally present both in the the serum and at the cell surface. The presence of a large number of free sulphydryl residues helps to explain the existence of dimeric or multimeric forms (49). This molecule has been studied intensively and a number of domains identified including a heparin binding site, a cell binding domain, and a collagen binding region (Figure 5) (50). A newly described family of integral transmembrane proteins known as integrins, has been shown to transduce fibronectin mediated extracellular signals to actin

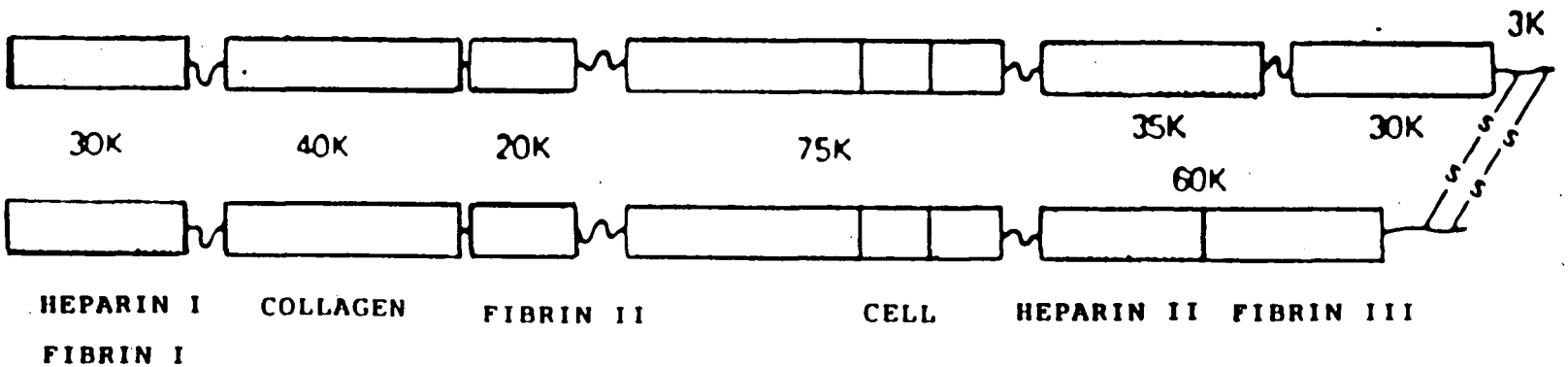

FIGURE 5. Functional Domains of Fibronectin.
Each box is a protease resistant functional domain.
Size of the domains are indicated by the numbers,
e.g. 75K = an apparent molecular weight of 75,000.
The binding activities of each domain are listed underneath.
The amino terminus portion of the molecule is on the left, and
the carboxy terminus on the right, adjacent to the disulphide
bridges.

filaments inside the cell (51). This suggests an attractive mechanism by which various extracellular matrices may interact with cells and eventually alter cardinal cellular functions such as gene transcription. A novel function of the fibronectin molecule has been put forth by Patel et al (52). These authors provided evidence that the release of mature red blood cells from the interstitial matrix of the marrow is mediated by the fibronectin molecule. A similar model has recently been proposed for granulocytic cells (53).

Hemonectin. Hemonectin is a very recently described marrow-specific adhesion molecule of a relative molecular mass of 60,000 daltons, that is immunologically distinct from any of the known extracellular matrix components (53). Immature granulocytes bind firmly to hemonectin, in contrast to mature granulocytes and granulocytic progenitors. This suggests that the loss of adhesiveness to this molecule may be part of the mechanism by which maturing neutrophils become able to leave the marrow.

Proteoglycans. Proteoglycans are long molecular complexes composed of a protein core to which a number of repeating disaccharide units are attached (54,55). With the exception of hyaluronic acid, these complexes are invariably acidic and can transform their immediate environment into hydrated gels. Recently, it has been shown that glycosaminoglycans (GAG's) produced by marrow cells in vitro can retain hemopoietic growth factor molecules (56). Further experiments are required to delineate the exact role and significance of this interesting phenomenon to the regulation of hemopoiesis in vivo.

2) REGULATION OF THE HEMOPOIETIC SYSTEM

Although still poorly understood, the regulation of many aspects of hemopoietic cell proliferation and differentiation appears to be mediated by a set of local environmental conditions and by the production of a number of hormone-like glycoprotein growth factors.

(A) Cellular Interactions

There are several lines of evidence suggesting that stromal cells of the bone marrow interact directly with hemopoietic cells and play a role in their regulation.

Ultrastructural Studies. Morphologic studies of the marrow have disclosed a number of consistent and specific interactions between hemopoietic cells and the various stromal populations (38). For example, an intimate relationship between adventitial reticular cells and hemopoietic cells has been noted. This has suggested that some physiologic functions are mediated through these interactions, possibly including the release of granulocytes from the marrow. Another intriguing finding has been the typical parasinal location of megakaryocytes. This observation is the basis of the proposal that endothelial cells elaborate hemopoietic growth factors capable of regulating or supporting megakaryocytopoiesis (57).

Regeneration Studies. A number of experimental strategies designed to disturb stromal cell support including local irradiation (58,59) and

mechanical disruption (60) have demonstrated that such treatments result in loss of hemopoietic function.

Studies of Spleen Colonies. It has been shown that the cellular composition (erythroid vs granulopoietic) of individual spleen colonies varies with their specific location in the spleen. Colonies arising under the spleen capsule or in the vicinity of septae are more often granulopoietic whereas in all other locations colonies are mainly erythroid. In contrast, colonies in the marrow are almost exclusively granulopoietic (E:G = 0.1). These observations suggest that local environments in these hemopoietic organs exert a profound influence on hemopoietic cell differentiation (61). Further, when marrow fragments were implanted into the spleen of experimental animals, which were then used as spleen colony assay recipients, the colonies produced in the intra-splenically implanted marrow stroma yielded a E:G ratio of 0.1 whereas those that developed in the spleen itself yielded an E:G ratio of 2.9 or higher. Colonies that bridged the junction of the spleen and the implanted marrow stroma had their erythroid portion in the splenic stroma and their granulopoietic portion in the marrow stroma (62). Although these studies cannot be construed as evidence of an "inductive" microenvironment in the deterministic (i.e commitment) sense of the word since they do not provide information as to the stage of hemopoietic cell development influenced, they do support the concept that fixed elements of hemopoietic tissues regulate some aspects of hemopoietic cell differentiation.

Transfer of the Hemopoietic Microenvironment. A series of studies undertaken by Friedenstein et al. (63) have shed considerable light on the role fixed bone marrow populations play in providing an environment capable of

supporting hemopoiesis. The studies of this group have shown that heterotopic transplantation of bone marrow fragments under the kidney capsule of semi-syngeneic animals leads to the formation of bone which then rapidly becomes populated by hemopoietic cells of recipient origin (63). In addition, transplantation of cloned, cultured fibroblasts under the capsule of the kidney was shown to result in the transfer of a microenvironment typical of the hemopoietic tissue from which the fibroblasts were obtained (64). Other groups have confirmed and expanded these observations and found that even subcutaneous implantation of a small amount of acellular diaphyseal bone extract can lead to the formation of a bone enclave which only then becomes populated with hemopoietic cells (65). Irradiation of the subcutaneous area, however, prevents the formation of the bone enclave, indicating that recruitment of subcutaneous cells is essential for the creation of a stroma suitable for hemopoietic stem cell invasion and support. Such studies have led to a model in which it is envisaged that undifferentiated "fibroblast-like" mesenchymal cells present in a variety of tissues can regenerate the differentiated elements of the hemopoietic stroma under the influence of particular, but as yet undefined extracellular matrix substances (66). Additional evidence in support of this is provided by recent co-culture studies showing that even 3T3 cells, which are undifferentiated cultured embryonic mouse fibroblasts can support hemopoiesis in vitro for a limited period of time (67).

Studies of the Hemopoietic Microenvironmental Defect in Mice Determined by the Sl/Sl^d Genotype. Mice bearing alterations at the Steel locus exhibit a number of abnormalities including macrocytic anemia, hypopigmentation, abnormal sensitivity to radiation, and sterility (68). Mutants of the

genotype Sl/Sl^d have been the most thoroughly studied and experiments with these animals have revealed the nature of the defect that affects their hemopoietic system. Hemopoietic stem cells in Sl/Sl^d mice are intrinsically normal as judged by their radiosensitivity, their ability to form spleen colonies in normal recipients and their ability to cure anemic mice whose hemopoietic stem cells are defective (69). However, when Sl/Sl^d animals are irradiated and then injected with normal (+/+) hemopoietic cells, they do not support hemopoietic recovery from the transplanted cells. This can be seen by the failure of the transplanted cells to form spleen colonies in Sl/Sl^d recipients (69). Since the hemopoietic defect can be cured by a graft of whole tissue from a +/+ mouse but not by an intravenous injection of suspended +/+ cells, the hemopoietic defect in the Sl/Sl^d mouse is thought to be due to a fixed cell of the marrow microenvironment that does not circulate and is not derived from a cell that does circulate (70).

Regulation of Stem Cell Turnover in Partially Irradiated Mice. Under normal conditions in the adult, hemopoietic stem cells are quiescent (G_o) or alternatively, have entered a very long cell cycle (2). However, in response to cytoreductive agents or radiation, a larger proportion of these cells are triggered to enter the S phase of the cell cycle. To determine if their turnover is regulated at a local level, experiments were performed in which mice were given whole body radiation, except for one tibia which was shielded. Using the ^{3}H-thymidine suicide assay (71), the cycling status of CFU-S was then followed both in the shielded and in the irradiated tibias. After 5 days, CFU-S in the shielded areas had largely returned to a quiescent state (12% kill). In contrast, in the irradiated limb the proportion of cells in S-phase was high (35 % kill) and the CFU-S content low. This disparity

between the two limbs clearly indicates that the turnover of primitive hemopoietic cells can be regulated locally (72).

(B) Humoral Regulation of the Hemopoietic System

Hemopoietic Growth Factors. The development of culture techniques capable of supporting the clonal growth of hemopoietic progenitors led to the identification and purification of a number of glycoproteins that are now collectively referred to as hemopoietic growth factors (HGF) (73). At first, biological activities present in crude preparations were operationally defined in terms of their ability to support colony formation, and hence were referred to as colony stimulating activities (CSA). The related, and now more widely used term, colony stimulating factor (CSF), was coined to refer to discrete purified factors. Only after such preparations were obtained could the full range of activity of individual CSF's be rigorously defined. Recently, the genes for a number of human HGF's have been cloned and expressed. These include: Erythropoietin (Epo), GM-CSF, G-CSF, M-CSF, and Interleukin-3 (IL-3). The availability of large quantities of these pure recombinant molecules has made it possible to investigate their in vivo biological activities in both primates and in man (74,75,76,77)) and to investigate their mechanisms of actions, in addition to defining the range of their activities on many different cell types.

Several points of interest have emerged from such studies: 1) HGF are glycoproteins which specifically interact with cell surface receptors present on target cells and their biological effects are often obtained even at a low (10%) receptor occupancy rates (78). 2) HGF are extremely potent molecules and are biologically active at picomolar concentrations (78). 3) There are no

striking homologies between the various HGF and any oncogene products thus far identified. However, the receptor for M-CSF has been shown to be identical to the product of the proto-oncogene, c-**fms** (79). 4) Most HGF have pleiotropic effects and can interact with a number of different target cells. These include mature effector cells which may have their functions augmented by the HGF's (80,81,82) as well as progenitor cells on different lineages (83).

Erythropoietin (Epo). Epo is a glycosylated glycoprotein with a relative molecular mass of 39,000 daltons when fully glycosylated. Native epo is produced mainly by the kidney and has been purified to homogeneity from the urine of patients with aplastic anemia (84,85). Epo is active on committed erythroid progenitors and enhances the survival and proliferation of CFU-E and mature BFU-E (86,87). The gene for human Epo has been mapped to chromosome 7 (88). Recombinant Epo has recently been tested in patients suffering from anemia of renal failure. The fully glycosylated recombinant molecule retains its full biological activity and shows great promise for the therapy of these patients (89).

Macrophage Colony-Stimulating Factor (M-CSF). Human M-CSF is a glycoprotein of a relative molecular mass of 47,000 to 76,000 daltons. This contrasts with the 70,000 dalton M-CSF purified from murine L cells. Upon reduction, the latter species yields 2 identical but biologically inactive subunits of about 35,000 daltons each (90). The gene has been cloned (91) and on the basis of its transcripts, 2 distinct forms have been postulated: a membrane-bound form and a secreted form, both of which are presumed to be biologically active (92,93). The proliferative effect of M-CSF on macrophage progenitors in vitro has been the best studied action of M-CSF (94). The human

gene for M-CSF is located on the long arm of chromosome 5 near the gene for the M-CSF receptor, **c-fms**.

Granulocyte Colony-Stimulating Factor (G-CSF). G-CSF was first purified to homogeneity from medium conditioned by the human bladder carcinoma cell line 5637 (95). It has a relative molecular mass of 19,600 daltons. The gene for human G-CSF is located on chromosome 17 (96). It is produced by a variety of activated cells including lymphocytes and fibroblasts (97,98). Recombinant G-CSF has been shown to support granulocyte progenitor cell proliferation in vitro (99). Although initial studies with purified natural human G-CSF suggested that this molecule could stimulate cells on other lineages, as well as pluripotent cells, hence the original term "pluripoietin" (95), more recent data suggest that these latter effects were primarily indirect (100). Interestingly, murine G-CSF, unlike most other murine HGF's, is active on human as well as murine cells and enhances the formation of human neutrophil colonies (101). An in vivo effect of human G-CSF in stimulating granulocyte levels in chemotherapy treated patients has been demonstrated very recently (75).

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF). GM-CSF is a glycoprotein of a relative molecular mass of 22,000 daltons. The human gene has been mapped to the long arm of chromosome 5 near M-CSF, **c-fms** and adjacent to IL-3 (102). GM-CSF, like G-CSF and IL-3, is not known to be constitutively produced by any normal cell but is produced by a variety of activated cells including lymphocytes and fibroblasts (103,104). Purified natural and recombinant molecules share the same biological properties: they both stimulate granulocyte, macrophage, eosinophil and megakaryocyte progenitors,

probably directly (105,106). They can also enhance the proliferation of primitive BFU-E and CFU-GEMM (107). The recombinant molecule has recently been tested clinically and a stimulation of circulating granulocyte levels in patients injected with GM-CSF has been demonstrated (76,77). In addition, GM-CSF can augment the function of mature effector cells and can stimulate the expression of cell surface adhesion molecules Mol and LeuM5 (p150,95) on mature granulocytes (81).

Interleukin-3 (IL-3), Multi-CSF. Human IL-3 is a glycoprotein with a molecular mass of 14,000 to 28,000 daltons depending on the extent the final molecule has been glycosylated. Although the existence of human IL-3 was predicted from studies of murine IL-3, human IL-3 was first obtained as a purified molecule as a result of an expression cloning strategy (108). The molecule has a large spectrum of activities, as predicted from studies of murine IL-3 (109), which appears to extend from the pluripotent stem cell compartment to the various mature committed progenitors. In contrast to GM-CSF, IL-3 is a more potent stimulator of erythroid bursts and mixed colonies and less potent stimulator of granulocyte-macrophage colonies (110).

Synergistic Activities. There have been a series of reports describing factors that, alone, are devoid of intrinsic colony-stimulating activity but, nevertheless, are capable of synergizing with one or more HGF's to enhance hemopoietic colony formation. Listed below are several such recently described synergistic factors.

Interleukin-1 (IL-1). Two forms of IL-1 have been described: IL -1α and IL-1β. Both are very similar, bind to the same receptor and, hence, share

most if not all of their biological effects (111,112). Il-1β is the predominant type expressed; however, an activity independently purified from 5637 conditioned medium on the basis of its ability to synergize with other HGF's turned out to be IL-1α (113,114,115). IL-1β is a polypeptide of a relative molecular mass of 22,000 daltons. It is produced mostly by activated monocytes (116) and TNF-α stimulated endothelial cells (117). It has a broad range of effects in vivo including the stimulation of secretion of some acute phase proteins (116), the acceleration of bone resorption (118), the enhancement of extracellular matrix protein turnover, and the induction of a variety of mesenchymal cell types (104,119,120) to secrete several growth factor molecules including: PDGF, nerve growth factor (NGF), and various CSF's (121,122,123,124,125,126). IL-1 has been shown to act on many cell types including keratinocytes, hypothalamic cells, hepatocytes, lymphocytes and mature neutrophils (120).

Interleukin-4/B Cell Stimulatory Factor-1 (IL-4)/(BCSF-1). A recently purified T cell product, murine IL-4, has been shown to stimulate a broad range of murine hemopoietic progenitors as well as B cells and T cells (127). However, these proliferative effects of IL-4 on myeloid cells are only observed in the presence of additional growth factor molecules. In concert with other factors murine IL-4 can enhance the in vitro proliferation of CFU-GEMM, CFU-GM, BFU-E, CFU-E and CFU-Mk (128,129). Murine IL-4 has been cloned (130) but its effect on human myeloid cells has not yet been established.

Interleukin-6 (IL-6). Previously known as Interferon-β2 (IFN-β2) or hybridoma growth factor (131), this molecule has a molecular weight of

26,000 daltons. IL-6 is produced by a variety of cell types including fibroblasts and monocytes particularly after their activation (132). It supports the growth of certain B cell hybridomas and plasmacytomas and has recently been found to synergize with IL-3 to support the proliferation of primitive multipotential murine progenitor cells in culture (133). IL-6 is a weak stimulator of macrophage progenitors in vitro. IL-6, like IL-1, also stimulates the secretion of acute phase proteins by hepatocytes (134).

Negative Regulators of Hemopoiesis. There have been reports by several groups (135,136,137) suggesting that fractionated cell-free extracts obtained from resting murine hemopoietic tissues contain a murine hemopoietic stem cell inhibitor whose action is reversible. This activity is associated with a molecular species whose mass appear to be in the 50,000 to 100,000 dalton range. It is detected by its ability to protect S-phase murine CFU-S from the lethal effects of exposure to high specific activity ^{3}H-thymidine (71). Interestingly, the effects of this inhibitor can be competed off by a smaller size (30,000 - 50,000 daltons) stimulator obtained from regenerating murine hemopoietic tissues (138,139). In a preliminary experiment the extracted inhibitor was found to be effective in vivo (140).

TGF-β has also been shown to also act as a potent inhibitor of hemopoietic cell proliferation (141,142). TGF-β is a highly conserved homodimer of 25,000 daltons present in many tissues. It is particularly abundant in bone where it is present at a 100-fold higher concentration than elsewhere (142). Two very similar forms of TGF-β have been identified: TGF-β1 and TGF-β2 (142,143). Very little is known at present about the mechanism of action of TGF-β1 or 2 at the molecular level, although the gene has been cloned (144). Recent reports have suggested that TGF-β can increase the

incorporation of fibronectin and collagen into the extracellular matrix and can enhance the expression of cell adhesion protein receptors (145). HGF-dependent murine hemopoietic cell lines which express specific TGF-β cell surface receptors (K = 1-60 pM) are inhibited by TGF-β1 (145). Recent experiments in the Terry Fox Laboratory have further shown that TFG-β1 can inhibit the cycling of the most primitive myeloid progenitor cell types in both mouse and human marrow in a direct, selective and reversible fashion (146).

Mesenchymal Cell Activators. As mentioned above, many HGF's are now known to be produced by mesenchymal cells following their activation by specific molecular mediators often produced in inflammatory reactions. Some of these, such as IL-1 have been discussed above as they can also act as HGF's. Others with no known direct HGF activity are reviewed briefly below.

Platelet derived growth factor (PDGF). PDGF is a cationic glycoprotein of a molecular mass of 30,000 daltons. PDGF consists of 2 polypeptide chains, one of which is identical to the product of the cellular proto-oncogene, c-sis (147). PDGF is produced by both activated endothelial cells and tissue macrophages (148,149). Induction of PDGF is obtained with endotoxin, tumor necrosis factor-α, and phorbol esters. Platelets contain the highest levels of PDGF, but release of PDGF from the platelet is conditional on the stimulation of platelet adhesion (125). Connective tissue cells (fibroblasts, smooth muscle cells, glial cells and chondrocytes) which display a large number of high affinity cell surface PDGF receptors are thought to constitute the primary target of PDGF action. Upon binding to its receptor, PDGF triggers a cascade of events which culminate in the production of

extracellular matrix substances, and the secretion of a range of biological activities, including GM-CSF (149,150). PDGF is rapidly cleared from the circulation, and current evidence suggests that its turnover may be regulated locally by its attachment to cells or intercellular matrix components (151).

Tumor Necrosis Factor-α (TNF-α). Also known as cachectin, this molecule elicits hemorrhagic necrosis of tumors in recipient animals challenged with endotoxin (152). It is produced principally by activated monocytes which then secrete very large amounts of TNF-α (153). TNF-α is rapidly distributed via the circulation to normal target cells: the monocytes and endothelial cells (153). Human TNF-α has recently been purified, the gene cloned, expressed, and mapped to chromosome 6 (154). TNF-α has a wide spectrum of activities and mediates endotoxin-induced shock. TNF-α has also recently been shown to provide mitogenic signals for endothelial cells in vivo, and to promote the formation of capillary tube-like structures in vitro. Like IL-1β and PDGF, TNF-α is a potent inducer of mesenchymal cells and stimulates them to produce and release G-CSF and GM-CSF (155,156).

Summary. It is clear from the above discussion that the bone marrow is a highly heterogeneous organ. It consists of many cell types and a diversity of fibrous and non fibrous cell products. A large body of evidence suggests that the regulation of hemopoietic stem cells is mediated primarily by complicated local mechanisms involving stromal cells and their secretory products.

3) LONG-TERM BONE MARROW CULTURES: AN IN VITRO MODEL FOR HEMOPOIETIC STEM CELL REGULATION

Although in vivo studies add weight to the physiological relevance of any effects observed following a given pertubation, they are usually poorly suited to the analysis of specific cellular and molecular events that underlie particular responses. The establishment and validation of suitable in vitro models for many cellular and tissue systems have therefore been a major objective of much research in cell biology. In this respect the study of hemopoietic stem cell regulation is no exception. The first description in 1977 by Dexter et al (157) of culture conditions which allow murine pluripotent stem cells to be maintained for many months in the absence of exogenously provided HGF's represented a significant advance in this regard. For the first time, it became possible to investigate in vitro, a system which supported stem cell self-renewal and differentiation. Moreover, as revealed by subsequent studies discussed in more detail below, this culture system has many features of the bone marrow as it is constituted in vivo and appears to provide a useful model for analysis of the regulatory systems operative in humans as well as mice.

(A) Early Development of the Long-Term Marrow Cultures

In initial studies with this system (157) mouse marrow cells were first placed in culture at a concentration of 10^6 cells/ml in a medium supplemented with horse serum to allow the formation of a confluent adherent layer which took 2-3 weeks. These cultures were then "recharged" with a second, similar inoculum of mouse marrow cells. Half the medium was then removed and replaced

each week. At the same time the cells in the non-adherent fraction were simultaneously diluted in half, and the cells removed could be counted, stained and/or assayed for various types of progenitors. Under these conditions, it was found that CFU-S, CFU-GM and granulopoiesis could be maintained for very extensive periods of time (158). An important feature of this system was that no HGF's were added exogenously. When they were, the result was not a positive one but rather an acceleration in the rate at which hemopoiesis declined (157). This, together with the demonstration of the critical importance of the adherent layer (159), suggested that cell-cell interactions, perhaps similar to those occurring in the marrow in vivo had been re-established in this system.

During the next several years, a number of significant technical improvements were identified. The use of hydrocortisone as a supplement was particularly important because it made it possible to use almost any batch of horse serum rather than only rare, selected batches. In addition, it made it possible to initiate cultures with a single inoculum of marrow (40). These improvements, in turn, led to the successful establishment of long-term human marrow cultures (160,161).

(B) Long-Term Human Marrow Cultures

Long-term cultures in which granulopoiesis is maintained for at least 8 weeks can now be routinely initiated from a single innoculum of normal human marrow. (For technical details, see Chapter II).

Within 3 weeks of placing the marrow cells in culture, a confluent adherent layer is established. In it a variety of stromal cell types have been identified. These include cells with properties of fibroblasts (162),

endothelial cells (see Figure 6A and 6B) (163), adipocytes (164), smooth-muscle cells (165), and macrophages (158). Interspersed between these cells, foci of developing hemopoietic cells, descriptively referred to as "cobblestone areas" (160), can be seen. The adherent layer also contains a number of extracellular matrix components including: collagen I (162), collagen III (162), collagen IV (166), laminin (167), and fibronectin (168). Hovering over and derived from the adherent layer are loosely adherent and free-floating cells. Most of these are mature granulocytes and macrophages and their immediate precursors (169). In the human system, some clonogenic progenitors can also be detected in the non-adherent fraction, but the majority of such cells, particularly the more primitive ones, are found in the adherent fraction (169). To quantitate the hemopoietic progenitor content of the adherent layer requires sacrificing the culture to enzymatically dissociate the cells in this fraction so that a single cell suspension suitable for plating in semi-solid medium can be obtained. Suitable methods using either collagenase or trypsin have been developed for this purpose (169). Assessment of a series of cultures over time has shown that the hemopoietic progenitor content of the non-adherent fractions and the mature granulocytes and macrophages to which they give rise are maintained at roughly constant levels for up to 8 weeks in spite of the demi-depopulation of the non-adherent cells at each weekly medium change (Figure 7). Note that although granulopoiesis is supported to completion in this system, erythropoiesis is not. Thus, only the most primitive erythroid progenitors, the BFU-E, are continuously detected and the more differentiated elements including the CFU-E rapidly disappear. This is not surprising since Epo is not normally added to these cultures. In the murine system, a similar situation is found and, although addition of Epo alone is insufficient to

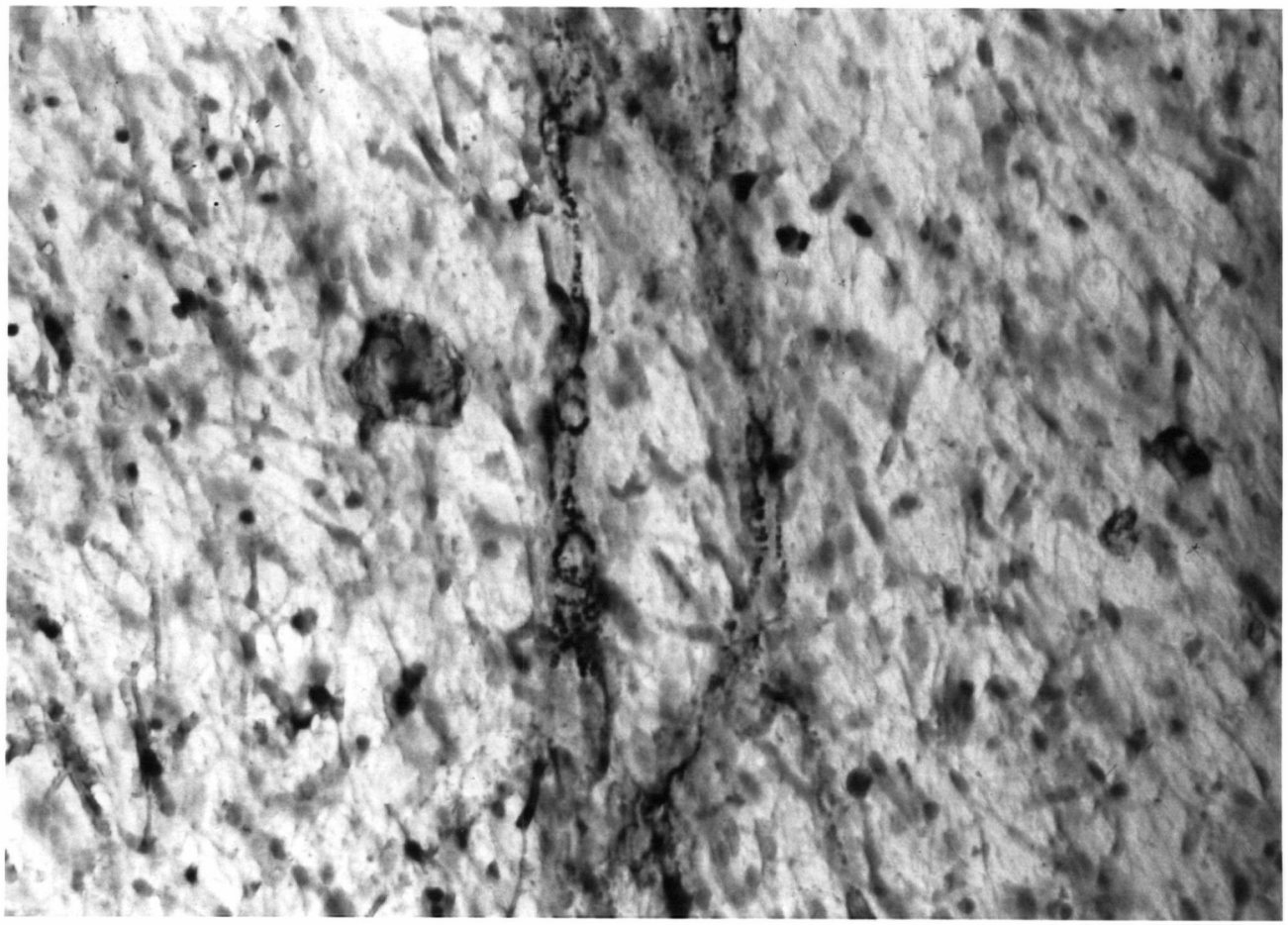

FIGURE 6. Photomicrographs of a Formalin Fixed Long-Term Marrow Culture Adherent Layer Stained With Rabbit-Anti Factor VIII Antiserum and Developed With Peroxidase-Labelled Swine Anti-Rabbit Immunoglobulin Antibody.

A) Low Power View

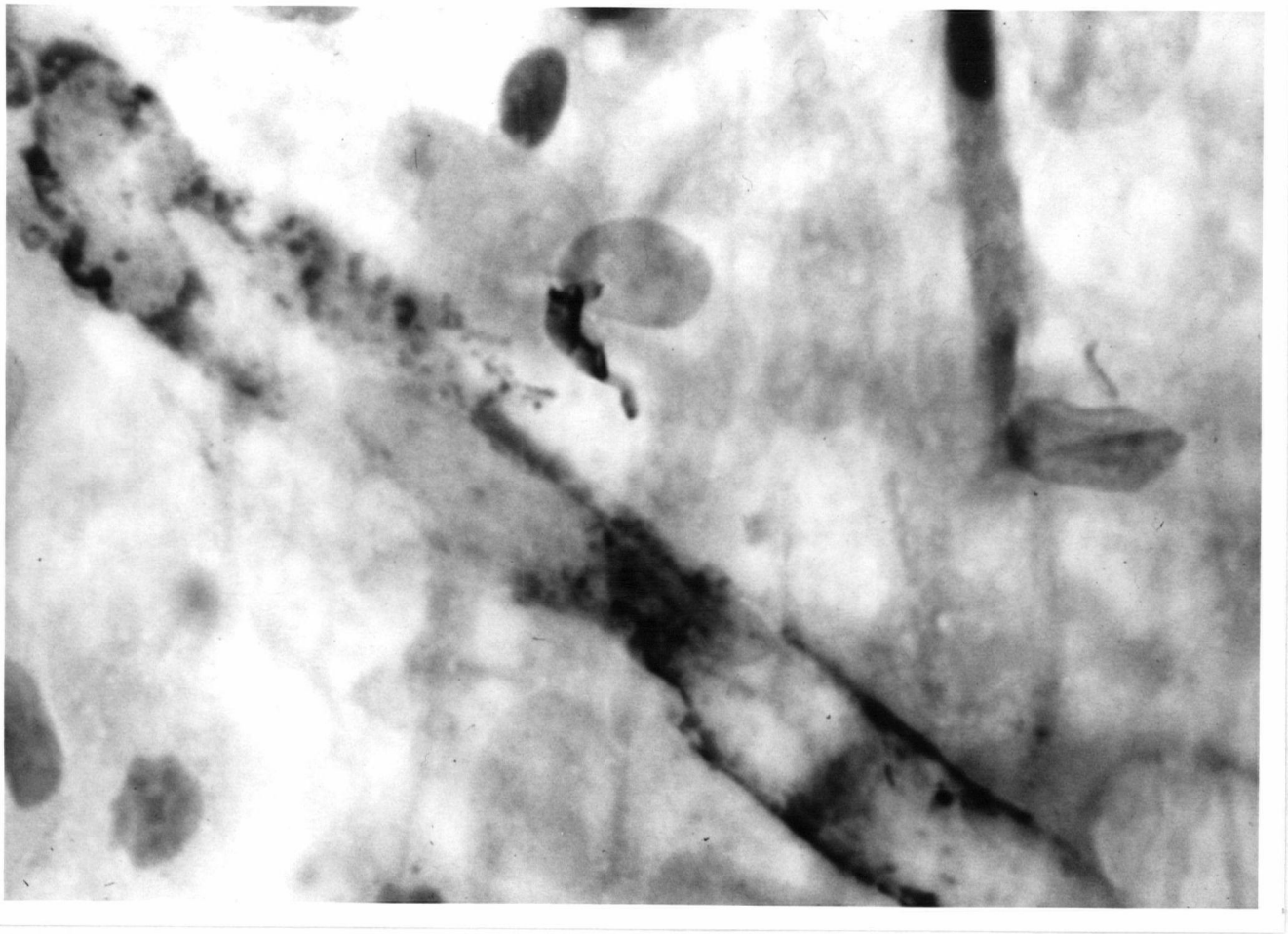

FIGURE 6. Photomicrographs of a Formalin Fixed Long-Term Marrow Culture Adherent Layer Stained With Rabbit-Anti Factor VIII Antiserum and Developed With Peroxidase-Labelled Swine Anti-Rabbit Immunoglobulin Antibody.

B) High Power View

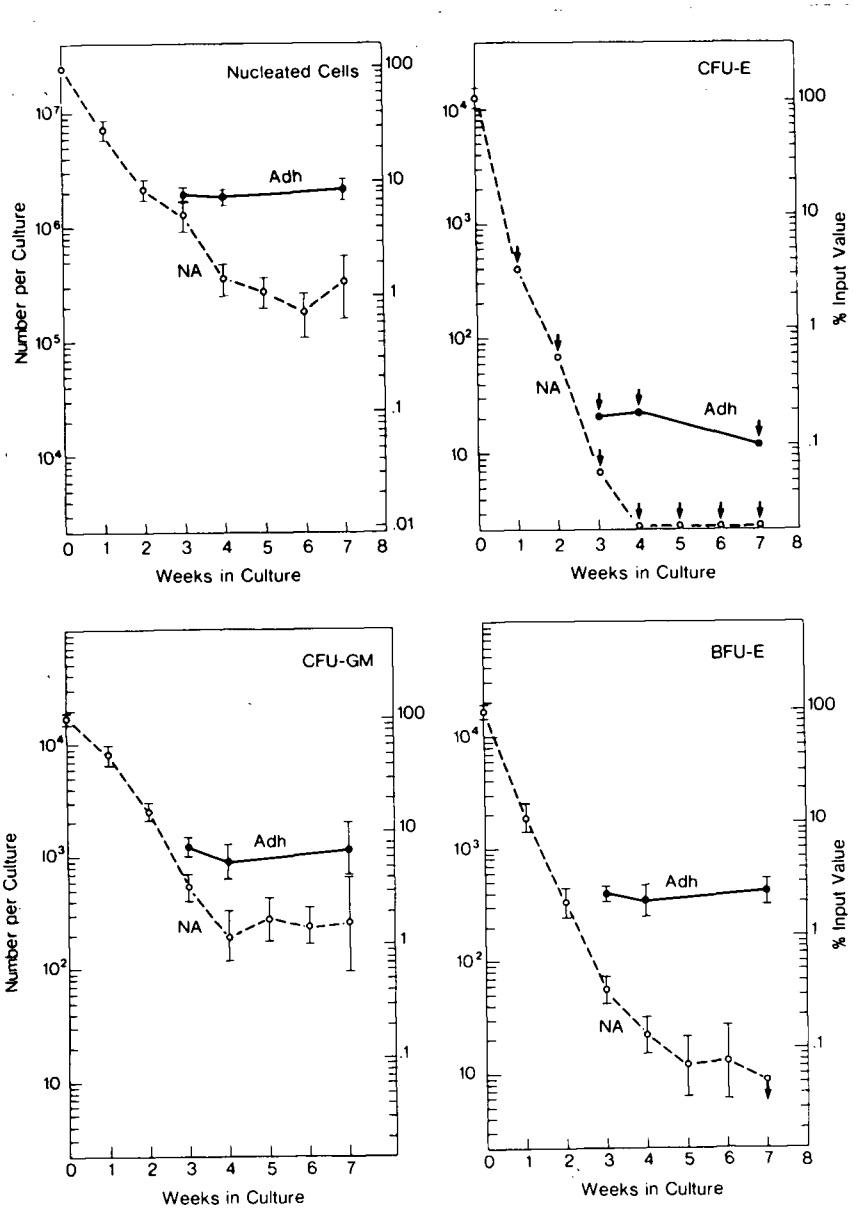

FIGURE 7. The Cellularity and Progenitor Content of the Adherent (Adh) and Non-adherent (NA) Fraction Assessed at Varying Incubation Times. Each point shown represents the geometric mean ± 1 SEM of data from several experiments. Of the 15 experiments initiated, five were terminated at 32 weeks, six at 4 weeks, and four maintained until 7 weeks and sacrificed for assessment at that time. The downward arrows indicate maximum mean values if one colony had been seen in any of the assay dishes scored in each individual experiment.

allow erythropoiesis to proceed, when other factors present in anemic serum are provided, massive production of mature red cells can be obtained (170).

(C) Long-Term Marrow Cultures as a Model of In Vivo Regulation

A number of studies in both the human and murine system have provided convincing evidence that the behaviour and regulation of hemopoietic cells in the long-term marrow culture system may reflect the operation of the same mechanisms that control hemopoiesis in vivo. This evidence may be summarized as follows: 1) In the murine system, where it is possible to assay cells for marrow repopulating potential by transplantation into lethally irradiated recipients, it has been shown that such cells are maintained in long-term marrow cultures (171). 2) When the mature differentiated granulocytes produced in long-term marrow cultures are compared with normal circulating peripheral blood granulocytes no differences in any of a variety of physiological properties, including phagocytosis, degranulation, respiratory burst and bacterial killing, are found (172). 3) The microenvironmental defect of the Sl/Sl^d mouse is reproduced in long-term cultures initiated with marrow from these mice (173). A normal appearing adherent layer is formed, but hemopoiesis is not maintained by comparison to cultures set up with marrow from +/+ littermates. Moreover, the defect can be overcome by seeding Sl/Sl^d marrow onto pre-established normal stromal cell layers from W/W^v mice (who have deficient stem cells and cannot initiate long-term hemopoiesis in vitro for this reason) (174). 4) Perhaps, the most compelling evidence comes from studies of the turnover of primitive hemopoietic cells in the long term marrow culture system. Assessment of the cycling status of different types of progenitors in the non-adherent and adherent fractions using the ^{3}H-thymidine

suicide technique to measure the proportion of S-phase cells (71) revealed a similar (although not identical) pattern in both murine and human cultures. In the murine system, CFU-S were found to oscillate between a cycling and non-cycling state dictated by the timing of perturbation of the cultures associated with each medium change. This resulted in an activation of all CFU-S within 1-2 days, regardless of their situation in either the adherent or non-adherent fraction. If the cultures were then left undisturbed, the CFU-S returned to a quiescent state within the next 3 to 5 days (175). In contrast CFU-GM were found to remain continuous in cycle as they do in vivo even under normal homeostatic conditions (175). In human marrow cultures, a similar pattern of alternating proliferation and quiescence in the most primitive progenitor compartments has also been seen (176). However, this behaviour is exclusive to the cells contained within the adherent layer, suggesting a more important role of direct cell contact in this species. This is further suggested by the finding that physical perturbation of human cultures is insufficient to achieve progenitor cell stimulation. Addition of some mesenchymal cell activator appears to be required (177). Nevertheless, as in the mouse, the cycling status of the various population of progenitors in unperturbed cultures closely mimics that seen in the marrow of the normal adult. Those classes of cells that in vivo are quiescent are those that in the long-term culture also return to a non-dividing state. Similarly, those that in vivo are in a state of constant turnover remain in cycle in the long-term culture system, even when they are located in the adherent layer.

4) THESIS OBJECTIVES

This research project was developed to explore the hypothesis that hemopoietic stem cell regulation is mediated by short-range interactions with mesenchymal elements. To test this hypothesis in the human, it would be advantageous to use an in vitro model in which the various components could be dissected out and their functions analysed separately.

In the preceding section I have described an in vitro system in which the most primitive hemopoietic populations detectable by clonogenic assays can be demonstrated for periods of at least 8 weeks. A key feature of this long-term marrow culture system is the development of a heterogeneous adherent layer of cells, including many of which are thought to resemble the various constituents of the microenvironment of the marrow in vivo. The availability of clonogenic assays for the detection of the most primitive classes of progenitors cells as well as the possibility of using the long-term marrow culture system as an in vitro model of the stroma suggested that further analysis of this system might allow the cellular and molecular mechanisms regulating stem cell maintenance and turnover to be identified. However, the long-term marrow culture model is by no means a "simple" version of in vivo hemopoiesis. The heterogeneity of the cellular composition of the adherent layer and the vast array of molecular species produced by these cells makes any proposed analysis a real challenge.

The overall objectives of my research were, therefore, twofold. The first objective was to develop a method for assaying the regulatory function of the non-hemopoietic components of long-term marrow culture adherent layers. Such an assay could then be used to evaluate the role played by individual stromal cell types. Prerequisite for such an assay was the need to obtain

pure target hemopoietic populations (or at least suspensions of hemopoietic cells that were free of regulatory cells). I envisaged that this might be achieved by appropriate physical separation techniques, or by the adoption of culture conditions that would selectively prevent contaminating accessory cells from interfering with the proposed measurements. Both approaches were explored and the results are presented in Chapter III.

The second objective of my research was to isolate homogeneous populations of cells corresponding to the various mesenchymal components of the marrow stroma. The approach was to establish permanent cell lines that could be cloned and then extensively characterized. It is well known that normal human mesenchymal cells senesce rapidly in vitro (178) and, in contrast to their murine counterparts, rarely become spontaneously immortalized. Therefore, an alternate strategy had to be used to immortalize human marrow stromal cells. Stimulated by reports of the ability of SV-40 virus to transform various types of human cells without loss of their differentiated phenotype (179,180,181,182), I initiated a series of experiments to try and obtain a series of human mesenchymal cell lines that exhibited properties of marrow stromal elements. The initial work involved in the preparation and titration of a suitable SV-40 virus preparation is described in Chapter II (Materials and Methods). The effects of this virus preparation on human marrow stromal cells and the characterization of the SV-40 transformed lines I isolated are described in Chapter IV.

Like most research projects, the achievement of these initial objectives turned out to represent definitive but relatively modest first steps towards the overall goal. Nevertheless, they made possible a clearer description of how next to proceed in unraveling the role of the marrow stroma. These are

discussed in the final chapter of this thesis under the title of Summary and Future Directions (Chapter V).

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CHAPTER II

MATERIALS AND METHODS

1. CELLS

(A) Bone Marrow Cells

Marrow aspirate cells were collected in heparinized medium with informed consent from hematologically normal patients undergoing routine investigations, or from normal allogeneic donors undergoing bone marrow harvests for transplantation. Light density mononuclear cells were obtained by centrifugation on Ficoll-Hypaque (1.077 gm/cm^3) and subsequently washed (3x) according to the directions of the supplier (Pharmacia, Dorval P.Q.).

(B) Peripheral Blood

Normal peripheral blood was obtained through the courtesy of the Canadian Red Cross. To isolate T-lymphocyte depleted mononuclear cells, a buffy coat preparation was first obtained by centrifuging whole units of peripheral blood diluted in acid/citrate/ dextrose at 800 x g for 5 minutes. Light density (<1.077 g/cm^3) mononuclear cells were then separated on Ficoll/Hypaque and T-lymphocytes then reduced to approximately 3-4% of total nucleated cells by incubation with 2-aminoethylisothiouronium bromide (AET, Sigma, St-Louis, MO.) treated sheep erythrocytes and removal of the T cell rosettes by re-centrifugation on Ficoll/Hypaque. This procedure yielded sufficient cells from 1 unit (500 ml) of blood to initiate 3 to 5 cultures with 2 x 10^7 cells each. Mean values from 14 experiments are shown on table I.

Table I. Recovery of Nucleated Cells and Progenitors from One Unit of Blood (500 ml)

Fraction Analysed	Yield (%)								
	Nucleated Cells	E Rosettes	CFU-E	BFU-E (3-8)	BFU-E (8-16)	BFU-E (>16)	BFU-E Total	CFU-GEMM	CFU-G/M
Unseparated Blood	100	-	-	-	-	-	-	-	-
Buffy Coat	49	-	-	-	-	-	-	-	-
Light-Density Cells	23	74	100	100	100	100	100	100	100
T-Depleted Cells	4	5	92	68	53	99	59	72	59
T-Enriched Cells	7	81	2	1	2	2	1	3	3

(C) Cell Line Maintenance

African Green monkey kidney cells (BSC-1), lung embryo fibroblasts (WI-38), and the human umbilical cord endothelial cell line HUV-EC-C were purchased from the American Type Culture Collection (Rockville, MD). Skin fibroblasts were isolated and established in vitro by Dr. D. Hogge also in the Terry Fox Laboratory. EAhy.926 cells were generously provided by Dr. C. J. Edgell (University of North Carolina, NC). The culture conditions used for the maintenance of these cell lines are summarized in Table II.

(D) Infection of Primary Human Mesenchymal Cells with SV-40 and Isolation of Permanent Cell Lines

Unless otherwise indicated, cultures to be infected were set up in triplicate in 60 mm tissue culture dishes, one of each containing 2 glass coverslips. The medium (α-10%) was removed and sufficient virus added to give 10^3 pfu/cell. In each case some cultures were not infected to serve as controls. After incubation at 37°C for 90 minutes to allow adsorption of the virus, the medium was changed. Twenty four hours later some coverslips were fixed and the proportion of adherent cells expressing the large T antigen was determined by indirect immunofluorescence (6). The remaining cultures were trypsinised, serial dilutions prepared, and 4 or more new cultures seeded from each dilution. Cultures were maintained at 37°C and fed twice weekly. One or two cultures were then reserved for isolation of individual transformed foci and others stained with May-Grünwald Giemsa. Individual foci were harvested

Table II. Culture Conditions for the Maintenance of Cell Lines

CELL LINE	ORIGIN	CULTURE MEDIUM
BSC-1 (1)	African Green Monkey Kidney	α-10%
WI-38 (2)	Human Embryonic Lung	α-10%
HUVE-EC-C (3)	Human Umbilical Cord Endothelium	F12 K Medium 20% FCS 100 µg/ml Heparin 50 µg/ml ECGS*
DH Fibroblasts (4)	Skin Fibroblasts	α-10%
EBV Lymphoblastoid Line	Human Marrow Cells in Long-Term Culture	α-10%
EAhy.926 (5)	Factor VIII +ve Mouse-Human Hybrid (A549xHUVEC-C)	α-10%
SV-40 Lines	Human Marrow and HUVE-EC-C cells (see section 1D)	α-10%

All cell lines were maintained in an atmosphere of 5% CO_2 at 37°C.

* Endothelial cell growth supplement.

using porcelain cylinders and the cells transferred to flat bottom tissue culture multiwell plates (Linbro, McLean, VA). A few days later, the lines were cloned by plating at limiting dilution in microtitre wells or by plating at 10^3 cells/35 mm dish in methylcellulose (see Anchorage-independent growth assays, section 7 below). Three weeks later, cells in positive wells or isolated colonies were removed and transferred individually to 2 cm^2 wells containing 2 ml of medium. Cloned lines were then propagated by feeding twice a week and subculturing once a week at 10^4 cell/cm^2 i.e. 2.5 x 10^5 cells/25cm^2 flask.

(E) Cell Proliferation Measurements

The growth and population doubling times of SV-40 infected cells and cloned lines were determined from viable cell counts (nigrosin dye exclusion) or from measurements of ^{3}H-thymidine incorporation. For measurements of population doubling time, cells were resuspended to a final concentration of 4 x 10^4 cells/ml. 2.5 ml of this cell suspension (i.e. 10^5 cells) was then seeded into 35 mm tissue culture dishes. At daily intervals thereafter, the cells in a pair of dishes (one infected and one control) were trypsinized and viable cell counts performed. To measure ^{3}H-thymidine incorporation, cells were cultured in Iscoves-10% in flat bottomed microwells (Costar) (500 cells/100 µl/well). At the times indicated, 1 µCi of ^{3}H-thymidine (20 mCi/mmole, Amersham, Oakville, Ont) in 20 µl of growth medium was then added to each well. The cells were incubated for an additional 4 hours at 37°C, harvested onto glass fiber filter paper, and the amount of ^{3}H-thymidine incorporated determined by scintillation counting.

2) LONG-TERM CULTURES

(A) Regular Long-Term Marrow Cultures

An aliquot of untreated marrow aspirate containing 2-2.5 x 10^7 nucleated marrow cells was placed in 8 ml of long-term culture growth medium in a 60 x 15 mm Falcon tissue culture dish. The growth medium was composed of α-medium supplemented with inositol (40 mg/l), folic acid (10 mg/l), extra glutamine (400 mg/ml), fetal calf serum (FCS, 12.5%), horse serum (HS, 12.5%), 2 mercaptoethanol (10^{-4} M), and hydrocortisone sodium succinate (10^{-6} M). FCS and HS were pretested for their ability to support maximal hemopoiesis in this system. The cultures were incubated for 3 to 4 days at 37°C in an atmosphere of 5% CO_2 in air. After this initial period of incubation, all nonadherent cells were removed and layered over Ficoll/Hypaque 1.077 g/cm^3 to remove remaining red blood cells and mature granulocytes where necessary. The light density cells were washed in α-medium supplemented with 2% FCS and returned to the original dishes. The cultures were then fed on a weekly basis by removal of half of the medium and half of the nonadherent cells. This was accomplished by pipetting 2-3 ml of medium from the dish, and then gently swirling the dish to ensure removal of all the nonadherent cells with the remaining 5-6 ml of the medium. The culture medium was placed in a tube, vortexed to distribute the cells evenly, and 4 ml of this suspension was returned to the culture dish, along with 4ml of fresh culture medium.

To harvest adherent layers, the cultures were first vigorously rinsed two or three times with serum free and Ca^{++} and Mg^{++} free, Hanks balanced salt solution to remove all the nonadherent cells (and any FCS left in dishes). The cells in the adherent layer were then exposed at 37°C to trypsin (0.25% in a

solution containing 5% citrate, 10% KCl and 1% glucose) for 10 minutes following the method of Coulombel et al. (7). Cells that were still adherent at the end of the incubation period were gently removed by pipetting, transfered to a test tube, centrifuged at 300 g x 10 minutes and washed twice in 2% α-medium.

(B) Long-Term Peripheral Blood Cultures

To initiate long-term peripheral blood cultures, 2 x 10^7 T-depleted light density mononuclear cells were placed in 60mm tissue culture dishes. These were subsequently maintained using the same protocol established for long-term cultures with the exception that cultures were incubated directly at 33°C and the day 3-4 red cell and granulocyte separation step was omitted. After 4-5 weeks, non-adherent cells were removed and then the adherent layer harvested by trypsinisation as described above.

(C) Preparation of Long-Term Marrow Culture Feeders

Long-term marrow cultures were initiated and maintained as described above with the following modifications. Hydrocortisone was omitted from the medium, the cultures were kept at 37°C, and at each medium change all of the nonadherent cells were removed and new medium added. Such cultures were used as feeders after the adherent layer had reached confluence (approximately 2 weeks), or in some cases after subculture and re-attainment of confluence in the secondary dishes. All feeders were irradiated with 15 Gy (see irradiation procedures, section 9, below) to ablate residual hemopoiesis, prior to addition of "test" cells. Assays of such irradiated pre-established adherent layers

consistently showed that they contained no detectable colony-forming hemopoietic progenitor cells.

(D) Cis-Hydroxy-L-Proline (CHP) Experiments

Long-term marrow cultures were initiated and maintained in the usual way, except that light density mononuclear cells rather than whole aspirate or marrow buffy coat cells were used to initiate the cultures, the step to remove cells >1.077g/cm^3 after 3-4 days was omitted, and cultures were placed from the beginning at 33°C. Cultures containing CHP (Sigma) were initiated and fed with a growth medium containing α-medium prepared without proline or lysine. Control cultures were maintained in the same medium but without CHP and with reconstituted levels of proline and lysine. After 4-5 weeks, nonadherent cells were removed and the adherent layer harvested by trypsinization. Some cultures were initiated by adding marrow cells into dishes containing pre-established irradiated feeders prepared as described above. These were handled in the same way as cultures set up in fresh dishes without feeders.

3) ASSAYS

(A) Methylcellulose Assay for Hemopoietic Colony-Forming Progenitors

Erythropoietic (CFU-E and BFU-E), granulopoietic (CFU-GM) and pluripotent (CFU-GEMM) progenitors were assayed by standard procedures previously described (7,8). Unless specified otherwise, cells were plated at a final concentration of 10^5 cells/ 1.1 ml of methylcellulose assay culture medium. Colony counts were obtained 3 weeks after plating from a minimum of 2 assay replicates except

in the case of fresh marrow and blood cell assays where CFU-E and mature BFU-E (erythroid colonies containing 1-2 and 3-8 clusters of erythroblasts, respectively) were scored after 1 1/2 weeks prior to final counts and other progenitors classes after 3 weeks.

(B) Colony-Forming Unit-Fibroblast (CFU-F) Assay

Essentially, the method described by Castro-Malaspina et al (9) was followed except that the medium used was α-medium made up with or without proline or lysine plus 10% fetal calf serum (FCS) to which varying concentrations of CHP or various growth factor preparations were then added, according to the experimental design. In brief, marrow buffy coat or light density (< 1.077 g/cm^3) cells were plated in tissue culture dishes at 8.5×10^2 cells/mm^2/2.6 µl or or 2.6×10^2 cells/mm^2/2.6 µl respectively, and then incubated undisturbed for 12 days at 37°C in a humidified atmosphere of 5% CO_2 in air. At the end of this time, the medium was decanted, the cultures rinsed twice in ice cold PBS, and adherent colonies fixed, air dried, stained with May-Grünwald-Giemsa.

(C) Assays for Production of Hemopoietic Growth Factor (HGF)

To test for Interleukin-1β (IL-1β) induced production of HGF, 2 ml of suspension of 2×10^4 cells/ml were placed into single wells of 24 multi-well plates (Linbro, McLean, VA) and 24 hours later, a small amount of purified recombinant human IL-1β (Biogen, Geneva) or diluent was added to give the desired final concentration of IL-1β . Conditioned media were harvested after

a further 24 hours, spun at 1,200 rpm for 10 minutes and the supernatant stored at -20°C.

To test if IL-1β stimulated SV-40 immortalized cell lines produced biological activity capable of stimulating marrow stromal cell progenitors I used the CFU-F assay with or without 10% IL-1β induced conditioned medium. As controls, we used growth medium alone (supplemented with 10% FCS), a crude source of hemopoietic growth factors (PHA-LCM 10%) and recombinant GM-CSF at a final concentration of 8 ng/ml. To initiate CFU-F assays I used the method described above with the exception that CHP was omitted and only regular α-medium supplemented with 10% FCS was used.

Colony-stimulating activity was tested by addition of conditioned media to standard methylcellulose cultures containing 3 to 5 x 10^4 non-adherent human marrow cells/ml. To reduce the background in negative controls (cultures containing no exogenous source of growth factor) and to obtain useful progenitor numbers at low cell concentrations, the target cells were obtained from the light density (<1.077 gm/cm^3) non-adherent cell fraction of 1 week-old long-term human marrow cultures (10). Positive controls included agar-stimulated leukocyte conditioned medium (LCM) and recombinant human GM-CSF (Biogen, Geneva). Unless specified otherwise, all cultures contained 3 units/ml of partially purified human urinary erythropoietin ($\geq$1,000 units/mg) (11).

4) SV-40 VIRUS PREPARATION AND ASSAY

(A) Preparation of High Titer Virus Stock

A freeze-dried preparation of SV-40 virus, strain A2895, was obtained from ATCC (Rockville, MD), reconstituted and the titre determined by plaque assay on confluent BSC-1 cells (6). A 200-fold higher titre stock was then prepared by infecting new, confluent BSC-1 monolayers at a low multiplicity of infection (0.01 pfu/cell) with the diluted lysate from a single plaque. The titre of the supernatant obtained from this infection was 8×10^{10} pfu/ml (see Figure 8). It was aliquoted into multiple vials and stored at -20°C.

(B) Virus Plaque Assay

The plaque assay described by Türler and Beard (6) was used. In brief, serial 10-fold dilutions of the virus were prepared in Tris-Dulbecco's buffer (6) supplemented with 2% FCS and 100 µl was used to infect confluent monolayers of African Green Monkey Kidney Cells (BSC-1 cells) in 60 mm tissue culture dishes. As controls, some were mock infected with 100µl of Tris-Dulbecco's buffer-2% FCS. After a 90 minute adsorbtion period, fresh 10% DMEM medium was added and the dishes were returned to 37°C for the next 15 hours. The medium was then replaced with 5 ml of 0.9% agar made up in the same medium. After the agar had set, cultures were then returned to the 37°C incubator for another 7 days. Plaques were counted using an inverted phase contrast microscope and titers (pfu/ml) of original virus stocks calculated.

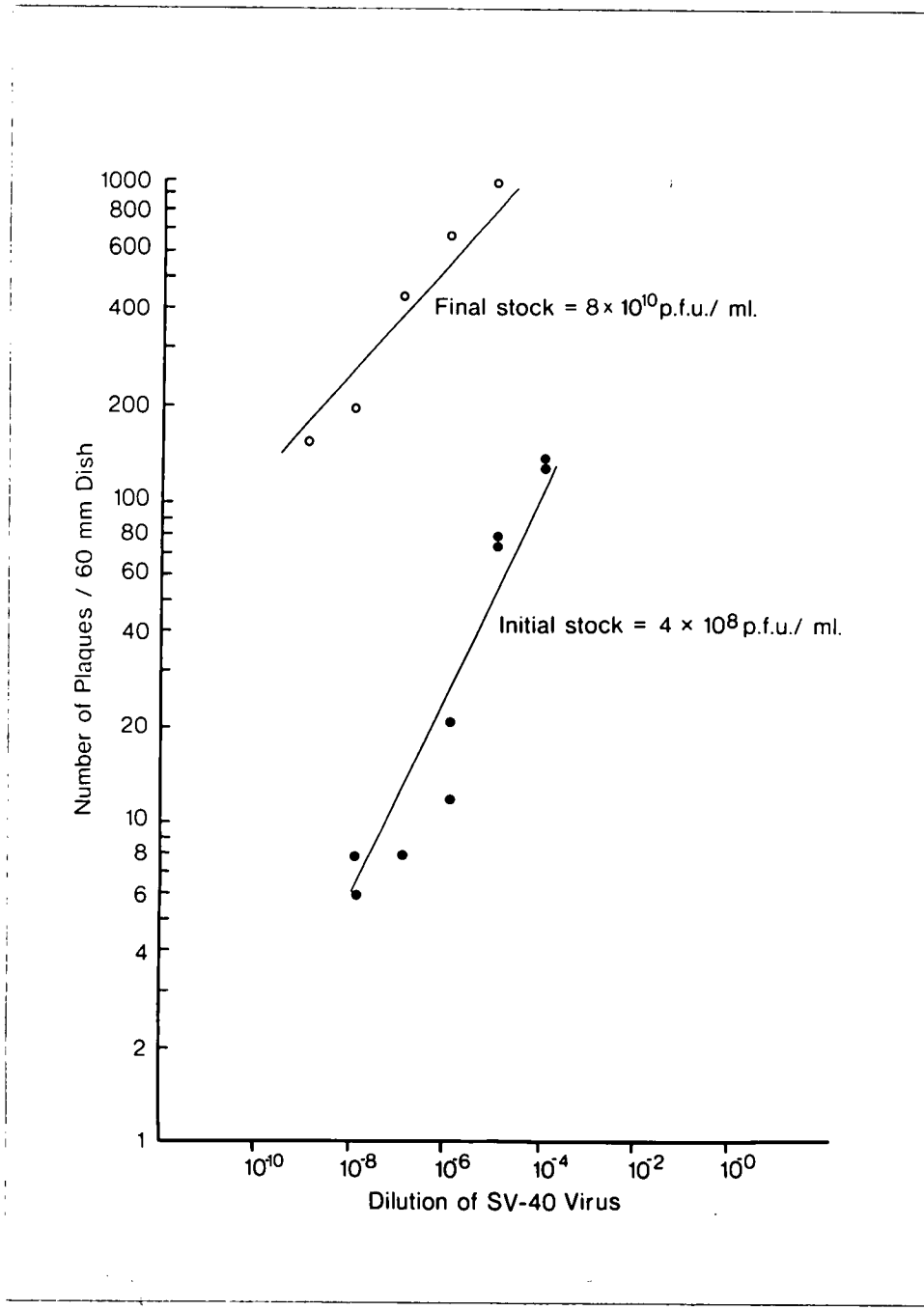

FIGURE 8. Titration of SV-40 Virus Stock on BSC-1 Cells.

(C) Assay For Large T Antigen

The presence of intranuclear T-antigen was detected by immunofluorescence using the highly specific monoclonal Pab 1626 (12). The method is fully described in section 5.

(D) Transformation Assay of SV-40 Virus on Mouse NIH-3T3 Cells

Freshly established NIH-3T3 cells were seeded in 60 mm dishes (Falcon) at a concentration of 2 x 10^4 cells/ml in 4.5 ml of Dulbecco's MEM with 10% FCS. Twenty four hours later 0.5 ml of serially diluted virus was added with 20 µg of polybrene. The medium was changed the following day and plates examined for the presence of dense colonies of cells after another 2 weeks. To score colonies, plates were fixed in 10% formalin and stained with May-Grünwald-Giemsa.

5) ANTISERA AND IMMUNOFLUORESCENCE MEASUREMENTS

For immunofluorescence microscopy, cells were grown to subconfluence on 22 x 22 mm coverslips, fixed in acetone/methanol 1:1 (v/v) for 5 minutes at -20°C, and then air-dried for 10 minutes at room temperature. After rinsing in Hanks balanced salt solution supplemented with 2% FCS and 0.1% sodium azide (HFN), cells were covered with the first antibody (listed in Table III) for 1 hour. To control for non-specific binding, duplicates were incubated with HFN alone. After 3 successive washes in HFN, an appropriate second antibody (either FITC-conjugated goat anti-mouse immunoglobulin, (CBL, The Netherlands)

Table III. Origin, Specificity and Source of Antibodies Used for Immunophenotype Analyses

Name	Cellular Specificity	Origin	Source
anti-laminin	Epithelial and endothelial cells	Rabbit*	Dr. Furthmayr (Yale, New Haven)
anti-collagen IV	Epithelial and endothelial cells	Rabbit*	" "
anti-collagen I	Fibroblasts	Goat*	Southern Biotechnology Assoc. (Birmingham, AL)
anti-T200/LCA/anti-Leuk AH	Nucleated Hemopoietic cells	Mouse+	Becton-Dickinson Corp. (Mountain View, CA)
anti-leuM1 (CD 15)	Differentiated monocytes, granulocytes	Mouse+	" "
anti-leuM3 (CD w14)	Monocytes, macrophages	Mouse+	" "
anti-6.19[15]	A variety of cells including all mesenchymal cell types studied but excluding all hemopoietic cells	Mouse+	Dr. Christopher Frantz (Rochester, NY)
anti-Factor VIII	Factor VIII-related antigen	Rabbit*	DAKO corporation (Santa Barbara, CA)
Pab 1626[21]	SV-40 Large T antigen-positive cells	Mouse+	Ciba-Geigy Ltd. (Basel, Switzerland)

*Antisera.

+Monoclonal antibodies.

or FITC-conjugated rabbit anti-goat immunoglobulin, (Nordic, The Netherlands) both at a final dilution of 1/80 was added and the cells then incubated for 30 minutes at room temperature. After 3 further washes in HFN, cells were mounted in buffered polyvinylalcohol (13) and examined with a Zeiss photomicroscope equipped with UV-epiillumination. For each antibody, a minimum of 400 cells were evaluated. To detect surface antigens freshly trypsinized cells were stained in suspension using the appropriate indirect procedure and a brief exposure to propidium iodide (2 µg/ml) after the final wash, and then analysed with a Becton Dickinson FACS IV equipped with a log amplifier. As negative controls, cells were labelled with the second FITC-labelled reagent only, or with an irrelevant monoclonal antibody of the same isotype as the first reagent. To estimate the proportion of positive cells, the channel number where the negative control and the test sample curves crossed each other was first determined. The number of negative cells to the right of this channel (higher fluorescence values) was then subtracted from the number of cells in the test sample that also fell to the right of the crossover point.

For immunoperoxidase detection of Factor VIII related antigen positive cells, long-term cultures were set up in Labteck (Napierville, IL) slide dishes. However, equally good results were obtained with Eahy.926 or HUV-EC-C cell lines grown on regular 22 x 22 mm coverslips. Slides were lightly fixed (1 min.) in acetone-methanol (1:1), endogeneous peroxidase activity suppressed by fixation in methanolic hydrogen peroxide for 20 minutes, and slides incubated sequentially with the following reagents (Dako Corp, Santa-Barbara, CA): Normal swine serum, polyclonal rabbit anti-Factor VIII anti-serum, swine anti-rabbit anti-serum, peroxidase-anti-peroxidase complex. The reaction was next revealed following incubation with a mixture of substrate, 3-amino-9-ethylcarbazole, 0.3% hydrogen peroxide in water, and 0.1M acetate buffer,

pH 5.2. Slides were counterstained with Gill's hematoxylin (Fisher, Orangeburg, N.Y.), diped several times in ammonia water, rinsed, and mounted using glycerol gelatin dip.

6) HISTOCHEMICAL ANALYSES

Cells were grown to subconfluence on coverslips, air dried and then stained for alkaline phosphatase and acid phosphatase by established histochemical methods (14,15).

7) TESTS FOR ANCHORAGE-INDEPENDENT GROWTH

Trypsinized cells were resuspended in α-2% to a final concentration of 3×10^5 cells/ml. Ten-fold dilutions were prepared in some cases down to concentrations of 30 cells/ml. 0.3 ml of these cell suspensions were then added to 3 ml of α-10% made viscous by the addition of 0.8% methylcellulose (4,000 cps, Dow Chemical, Vancouver, B.C.). One ml cultures were plated in 35 mm dishes (Greiner, FRG) and incubated at 37°C in a fully humidified atmosphere containing 5% CO_2. Colonies containing more than 50 cells were scored 20 days later with an inverted microscope.

8) TUMOR FORMATION

A total of 0.1 to 1×10^6 SV-40 immortalized cells (MH2-SV) in 100 µl of saline were injected intradermally into 4 female nu/nu mice. As controls, 2 additional animals were injected with saline alone.

9) IRRADIATION PROCEDURES

For cocultivation experiments, preestablished adherent layers were irradiated with 15-20 grays (^{60}Co γ rays or 250 KVp X-rays). To determine the relative radiosensitivities of SV-40 immortalized lines and normal marrow stromal cells in culture, freshly established layers were trypsinised and washed twice in growth medium. Single cell suspensions were prepared and a fixed number (10^4-10^6) of cells dispensed in test tubes (Falcon) and kept on ice except during irradiation ($\leq$2 minutes). Radiation (250 KVp X-rays) was given at a single dose at the rate of 5.2 Gy/min. Cells were then resuspended in 7.5 ml of fresh α-20% tissue culture medium, dispensed in 60mm tissue culture dishes and incubated at 37°C for 10-14 days . To assess the effects of irradiation on colony forming cells the mean number of colonies formed was used to calculate the plating efficiency and their surviving fraction relative to the control cultures.

10) AUTORADIOGRAPHY

To determine the effects of SV-40 virus on contact-inhibited marrow adherent cells, 10^5 cells from a trypsinized 4 week old normal long-term bone marrow culture were resuspended in 2.5 ml of Dulbecco's MEM and seeded into 35 mm Falcon tissue culture dishes. Five days after reaching confluence, the medium was removed and cultures incubated with or without 250 µl of SV-40 virus stock (10^{10} pfu/ml). Twenty four hours later dishes were rinsed, covered with Iscove's medium supplemented with 15% FCS and returned to the incubator for one more day. Cell layers were allowed to incorporate ^{3}HTdR at a final concentration of 10µCi/ml and labelled nuclei subsequently visualized by

autoradiography. Cells were rinsed with Tris-buffered saline, fixed in 3.7% formaldehyde for 10 minutes, and washed sequentially with 5% trichloroacetic acid, water, and 70% ethanol. After drying, dishes were coated with Kodak NTB2 photographic emulsion, dried again, and left in the dark for 2 more days in a dessicating jar. The emulsion was then developed and the cells stained briefly with 2% methyl green. The percentage of labelled nuclei was then calculated.

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CHAPTER III

DIFFERENTIAL EFFECTS OF CIS-OH-L-PROLINE ON THE PROLIFERATIVE AND STEM CELL REGULATORY FUNCTIONS OF HUMAN BONE MARROW MESENCHYMAL CELLS

1. INTRODUCTION

There is now considerable evidence from both in vivo (1,2,3) and in vitro (4,5,6) studies indicating that fixed, i.e. non-circulating, mesenchymal elements are involved in the regulation of hemopoiesis in the marrow. Such cells are a major component of the adherent layer of long-term marrow cultures, which serves as an attractive model for in vitro analyses since it allows hemopoiesis to be maintained for extensive periods of time (months) in the absence of exogenously added hemopoietic growth factors. However, since the adherent layer of long-term marrow cultures consists of a diversity of cell types of both mesenchymal (e.g. fibroblasts, adipocytes and endothelial cells) and hemopoietic origin (e.g. macrophages and progenitors) (7,8,9) reconstruction experiments are needed to delineate both the nature and the cellular origin of the factors that needed for the maintenance and regulated turnover of primitive hemopoietic cells.

In the first part of my research, the main objective was to develop an experimental system that would allow direct measurements of the hemopoietic sustaining function of mesenchymal cells present in the adherent layer of long-term bone marrow cultures. A prerequisite for assaying such a function rests on the availability of a hemopoietic target population that is free of mesenchymal cells but nevertheless requires the establishment of close

interactions with non-hemopoietic stromal cells for long-term survival in culture.

Several strategies to obtain such populations were explored: Isolation of light-density non-adherent cells from hemopoietically active long-term bone marrow cultures (10), physical separation of fresh bone marrow cells on nylon wool columns (11), immunological purging of bone marrow cells with a combination of monoclonal antibody 6.19 and complement (12) and, modification of the culture conditions by the addition of D-valine to the medium (13). None of these approaches could reduce to acceptable levels the proliferation of residual mesenchymal cells present in unseparated marrows. A more successful approach utilized peripheral blood as a potential target population. Peripheral blood represents an attractive option since multipotential and high proliferative potential myeloid progenitor are present at a reasonable frequency whereas mesenchymal cells are totally absent (14,15). Thus, I investigated the possibility that progenitor cells from this source could be enriched in sufficient amount to initiate cultures on irradiated pre-established adherent layers.

In the second approach, I sought to determine if Cis-4-hydroxy-L-proline (CHP), a relatively specific inhibitor of collagen synthesis (16) could be used to selectively inhibit the mesenchymal cell component of marrow cell suspensions and therefore allow marrow to be used as a simple source of hemopoietic progenitors. Although preliminary results with appropriate monoclonal antibodies have suggested that highly purified hemopoietic progenitors can now be obtained, and these might then be of some value for such experiments (17), the use of CHP had the attraction that it was technically simpler and had the potential to avoid the tremendous cell losses and considerable variability typically encountered in cell separation experiments.

Recently, CHP was reported to prevent the formation of an adherent layer in murine long-term marrow cultures at doses that are not directly toxic to co-existing hemopoietic progenitor cells (18). In the present study, I investigated the possibility that CHP might have a similar differential effect on the proliferative and stem cell regulatory functions of human marrow mesenchymal cells. To address this question, I first evaluated the effect of CHP on the proliferation of collagen-producing cells from human marrow as assessed in both CFU-F assays and in the long-term marrow culture system. CHP proved to be an effective inhibitor of these cells at concentrations that are not toxic to human hemopoietic cells present in the same cultures. CHP supplemented medium should therefore be useful to enable unseparated marrow to be used to assess the potential ability of defined populations of mesenchymal cells to support the long-term maintenance and proliferation of human hemopoietic progenitor cells.

2) RESULTS

(A) Cultures of Peripheral Blood Cells on Normal Marrow Adherent Layer

Light density (<1.077 gm/cm^3) peripheral blood cells were depleted of T cells (to enrich for myeloid progenitors) and then used to initiate long-term cultures by seeding the cells into dishes containing a confluent, irradiated adherent layer from a previously established long-term marrow culture, or into new dishes without such a "feeder" as controls. The total numbers of nucleated cells and clonogenic erythroid and granulopoietic progenitors present in these cultures are shown on Figure 9. In the presence of a marrow adherent layer the number of non-adherent cells (panel a) and clonogenic progenitors

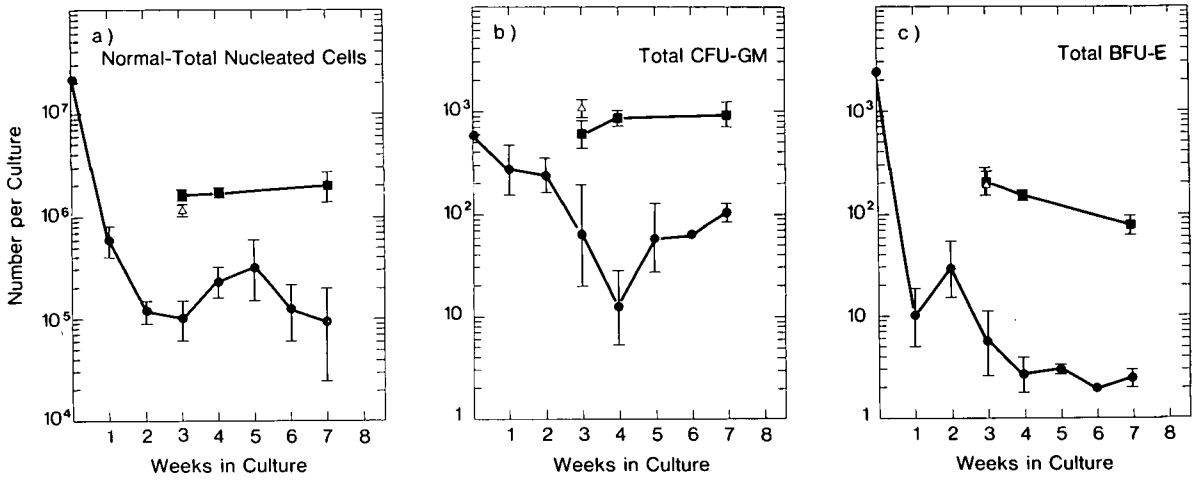

FIGURE 9. Comparison of Total Cell and Progenitor Content of Long-Term Normal PBL Cultures Initiated With (Solid Symbols) or Without (Open Triangles) a Pre-Established Normal Marrow Feeder. Circles - data for non-adherent fractions of cultures with feeders assessed weekly. Squares - data for adherent fractions of cultures with feeders. Triangles - data for non-adherent fractions of cultures without feeders (no adherent fraction obtained), from which no cells were removed at each weekly medium change until the culture was sacrificed and which were used for parallel cycling measurements (19). Values shown are the geometric means $\pm$ 1 SEM from data of 5 experiments.

(panels b and c) sharply declined during the first 2-3 weeks, but after 3-4 weeks a plateau phase was reached and thereafter the numbers remained relatively constant for the duration of the cultures. When these cultures were harvested at 3 weeks, the majority of the clonogenic progenitors were found in the adherent layer and values were sufficient to allow their proliferative status to be determined by Dr. J. Cashman (19). These studies revealed an alternating pattern of proliferation and quiescence of primitive but not mature progenitor cell types in the adherent layer as a function of the feeding schedule. Cultures that were initiated in the absence of a pre-established marrow adherent layer also contained sufficient progenitors at 3 weeks to allow cycling measurements to be performed. In these cultures all progenitors were non-adherent and in contrast to cultures with adherent layers all progenitors, including those classified as primitive were proliferating, irrespective of the feeding pattern. However, since the feeders had no effect on the number of progenitors present after 3 weeks, cultures had to be maintained for at least 5 weeks to enable a differential effect of the marrow adherent layers to be observed. This became impractical for routine experiments due to the emergence in a number of cultures of polyclonal B-lymphoblastoid populations originating from cells latently infected with Epstein-Barr virus, a finding also reported by others (20). These cells first became noticeable in methylcellulose cultures where they formed large (>10,000 cells), diffuse colonies which sometimes overgrew the entire culture. Microscopic examination of plucked colonies revealed a homogeneous population of lymphoblastoid cells with a high nucleo-cytoplasmic ratio, prominent nucleoli, and a small rim of basophilic cytoplasm. Cell markers studies were also performed on these colonies (see Table IV) and their B-cell origin confirmed. Because of this phenomenon I explored alternative sources of hemopoietic progenitors.

Table IV. Immunophenotypic Characterization of Lymphoblastoid Cells

Marker Assessed	% Positive
T-Cells	
E-Rosettes	0
Leu-4	1
α-Thy	1
B-Cells	
Leu-12	72
B4	59
λ	9
κ	31
Pan-Hemopoietic	
T-200	81

(B) Effect of CHP on Marrow Mesenchymal Cell Proliferation

This was evaluated in two different types of experiments, one using the CFU-F assay and one using the long-term marrow culture system. These differ both in the number of cells initially used to seed the cultures, and in the duration of time prior to assessment of the CHP effect. The experiments employing the CFU-F assay demonstrated a reproducible dose-dependent inhibition of fibroblast colony formation in cultures containing CHP (Figure 10). Additional treatment groups included in these experiments demonstrated that omission of proline and lysine from the medium had, on its own, no effect on the number or size of fibroblast colonies obtained in the absence of CHP and, conversely, that the restoration of the proline and lysine content of the medium to "normal" levels completely abrogated the CHP effect (data not shown). Concentrations of CHP above 50 µg/ml reduced marrow fibroblasts colony numbers to <50% of control values and at concentrations above 250 µg/ml, no fibroblast colonies were seen.

In the second set of experiments, the effect of CHP on adherent layer formation in the long-term marrow culture system was assessed. Preliminary experiments showed that CHP concentrations below 100 µg/ml, although significantly inhibitory in CFU-F assays, still allowed significant adherent layer formation in the long-term culture system when this was assessed after 4-5 weeks. However, when the concentration of CHP was increased to 500 µg/ml, the formation of an adherent layer was severely and irreversibly impaired. Table V shows the results of cell counts performed on adherent layers fromparallel cultures initiated and maintained in the presence or absence of CHP harvested after 4-5 weeks. The dramatic difference in the appearance of these cultures is illustrated in Figure 11.

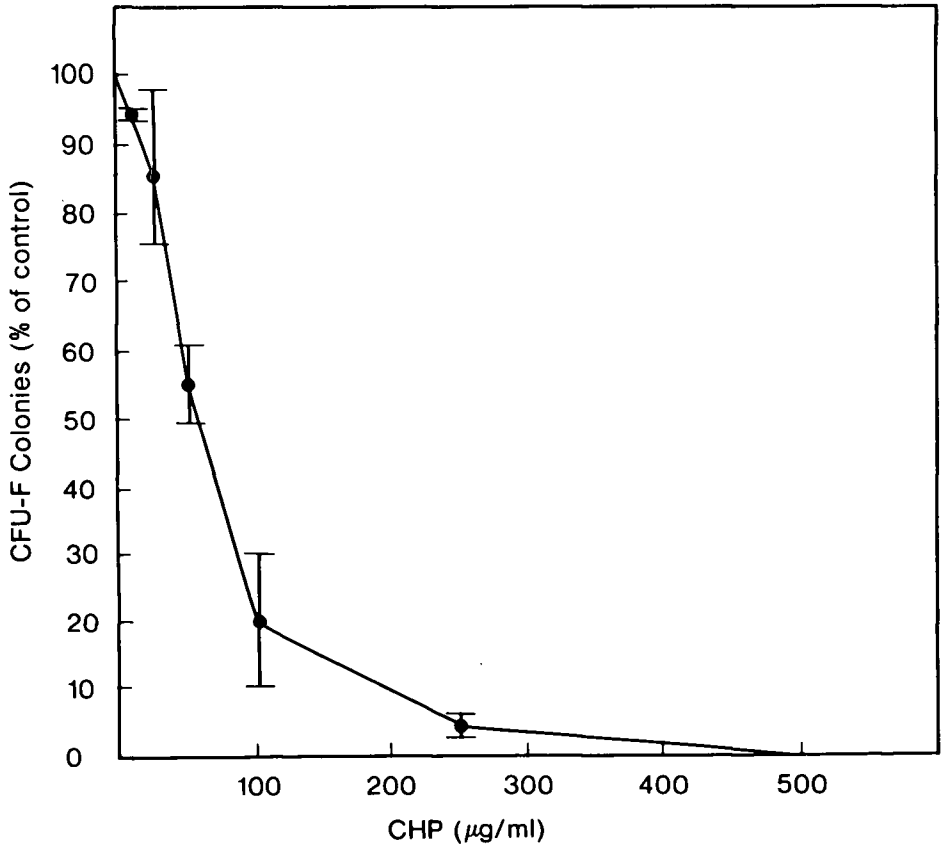

FIGURE 10. Effect of Increasing Doses of CHP on the Number of Colonies Obtained from CFU-F in Fresh Human Marrow. Values represent the mean ± 1 SEM for normalized data (% of number of colonies seen in the absence of CHP) from 5 different experiments (different marrow samples).

Table V. Effect of CHP on Adherent Layer Formation

Exp. no.	No. of cells in the adherent layer at 4-5 weeks ($\times 10^{-5}$)		
	- CHP	+ CHP (% of control without CHP)	
1	38.0	3.1	(8.2)
2	24.0	0.4	(1.6)
3	12.0	0.8	(6.7)
4	8.4	1.8	(21.4)
Mean $\pm$ SEM			9.5 $\pm$ 4.2

The difference between - CHP and + CHP is significant ($p<0.05$ using paired t-test (27)).

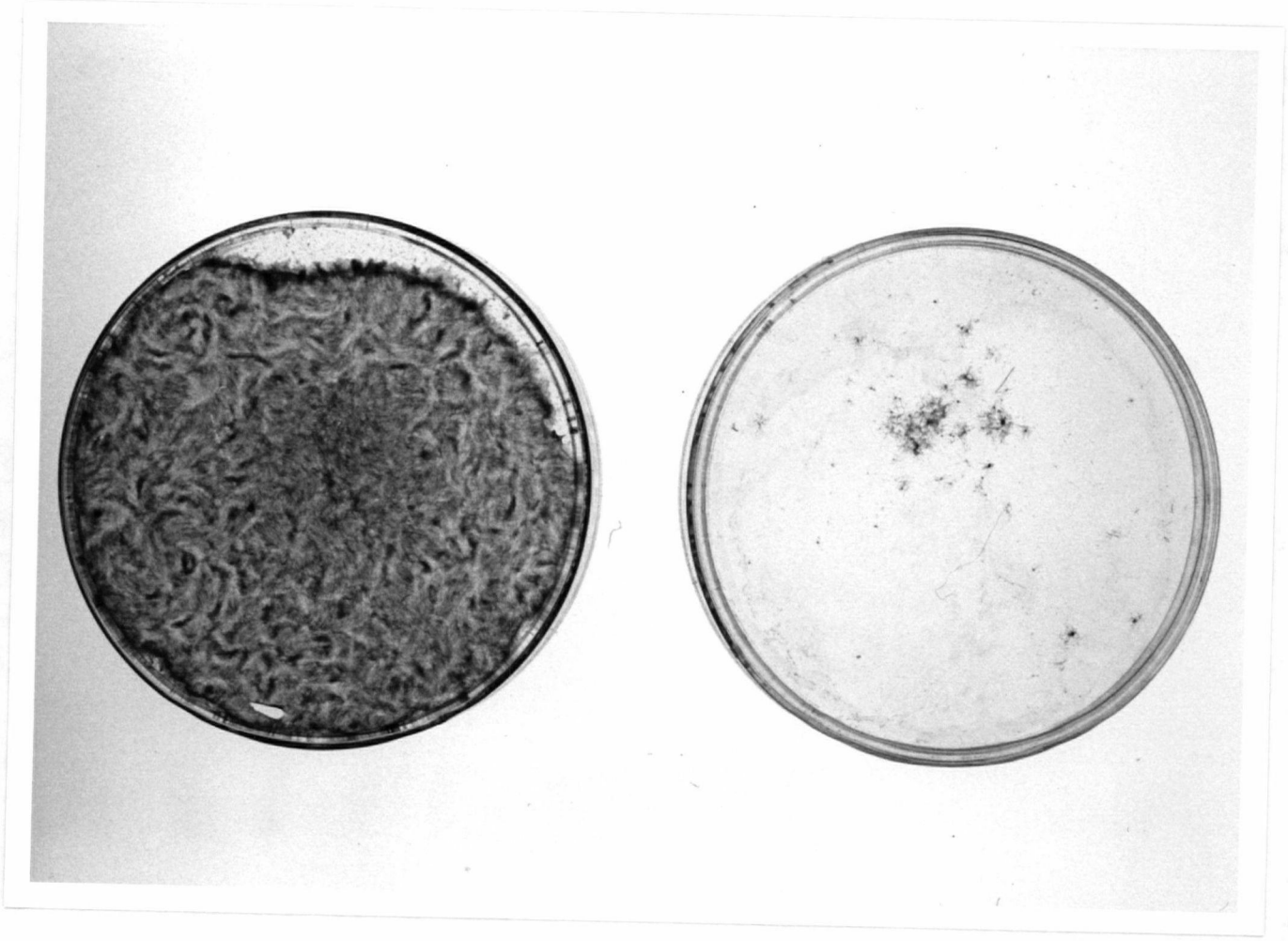

FIGURE 11. Photograph of Two Long-Term Marrow Cultures, One Initiated and Maintained in Regular Medium (Left), and One Initiated and Maintained in Proline-, and Lysine-Free Medium Containing 500 µg/ml of CHP (Right).

(C) Lack of a Direct Effect of CHP on Hemopoietic Progenitor Function

To investigate possible CHP effects on hemopoietic cells, marrow cells were first incubated for 3 hours in proline-, lysine-free medium with or without added CHP, and then washed and plated in methylcellulose assays. As shown in Table VI, such short-term exposure of marrow cells to even 500 µg/ml CHP had no effect on the number, size or composition of the colonies produced by any of the progenitor classes assessed.

The effect of a longer exposure of hemopoietic cells to CHP in the long-term marrow culture system was then evaluated. Since the presence of CHP in the medium was known to inhibit adherent layer formation, it was anticipated that hemopoiesis might also be reduced even if there were no direct effect of CHP on the hemopoietic cells themselves, since previous studies have indicatedthat the long-term support of hemopoiesis in this system requires an intact adherent layer (4,6,17,18). An additional group was therefore included to allow any direct effects of CHP on the production or survival of hemopoietic cells to be distinguished from potential indirect effects caused by CHP inhibition of mesenchymal cells that might be essential for the maintenance of the hemopoietic cells. Thus, the following three conditions were tested in each experiment. The first two compared the presence and absence of CHP in long-term marrow cultures initiated using the standard protocol, i.e. marrow cells were seeded into new dishes in medium with or without 500 µg/ml CHP. In the third group, marrow cells were maintained in medium containing 500 µg/ml of CHP, but the dishes into which they were seeded already contained a preestablished marrow adherent layer. This had been obtained by subculturing a

Table VI. Lack of Effect of Exposure of Hemopoietic Progenitors to CHP on Their Subsequent Plating Efficiency

Progenitor	Exp no.	CHP (μg/ml) 0	25	500
CFU-E	1	218	209	266
	2	163	125	91
	3	256	230	265
BFU-E	1	164	177	140
	2	88	87	55
	3	176	186	140
CFU-G/M	1	168	144	146
	2	80	93	82
	3	151	154	158
CFU-G/E	1	10	16	10
	2	4	3	4
	3	7	2	7

* Each value shown represents the mean value from 2 replicate assay cultures. Light density mononuclear marrow cells were incubated for 3 hours at 37°C in the presence or absence of CHP in lysine-, proline-free medium. At the end of the incubation period, cells were washed once and the equivalent of 10^5 of the original cells were plated in standard 1.1 ml methylcellulose assays.

previously established, regular long-term marrow culture and then irradiating the secondary adherent layer produced with 15 Gy to inactivate any residual hemopoietic progenitors still present (19). It has been our experience at the Terry Fox Laboratory that such subcultured adherent layers are equivalent to primary adherent layers in terms of their ability to support and regulate long-term human hemopoiesis, but are not associated with the same problems of adherent layer detachment frequently encountered when such co-cultures are set up using primary adherent layers. The number of progenitors present in each of these three types of long-term cultures was assessed after 4-5 weeks. The effect of CHP on progenitor maintenance in cultures established using the standard protocol (i.e. no feeders) is shown in Figure 12. It can be seen that, in the absence of a feeder, hemopoiesis was markedly inhibited in cultures containing CHP. However, when a pre-existing feeder was provided, hemopoiesis was equivalent to that observed in control cultures (no CHP, no feeders) (Table VII). Thus, the continuous presence of even 500 µg/ml of CHP does not appear to be directly detrimental to the survival, proliferation or differentiation of hemopoietic cells.

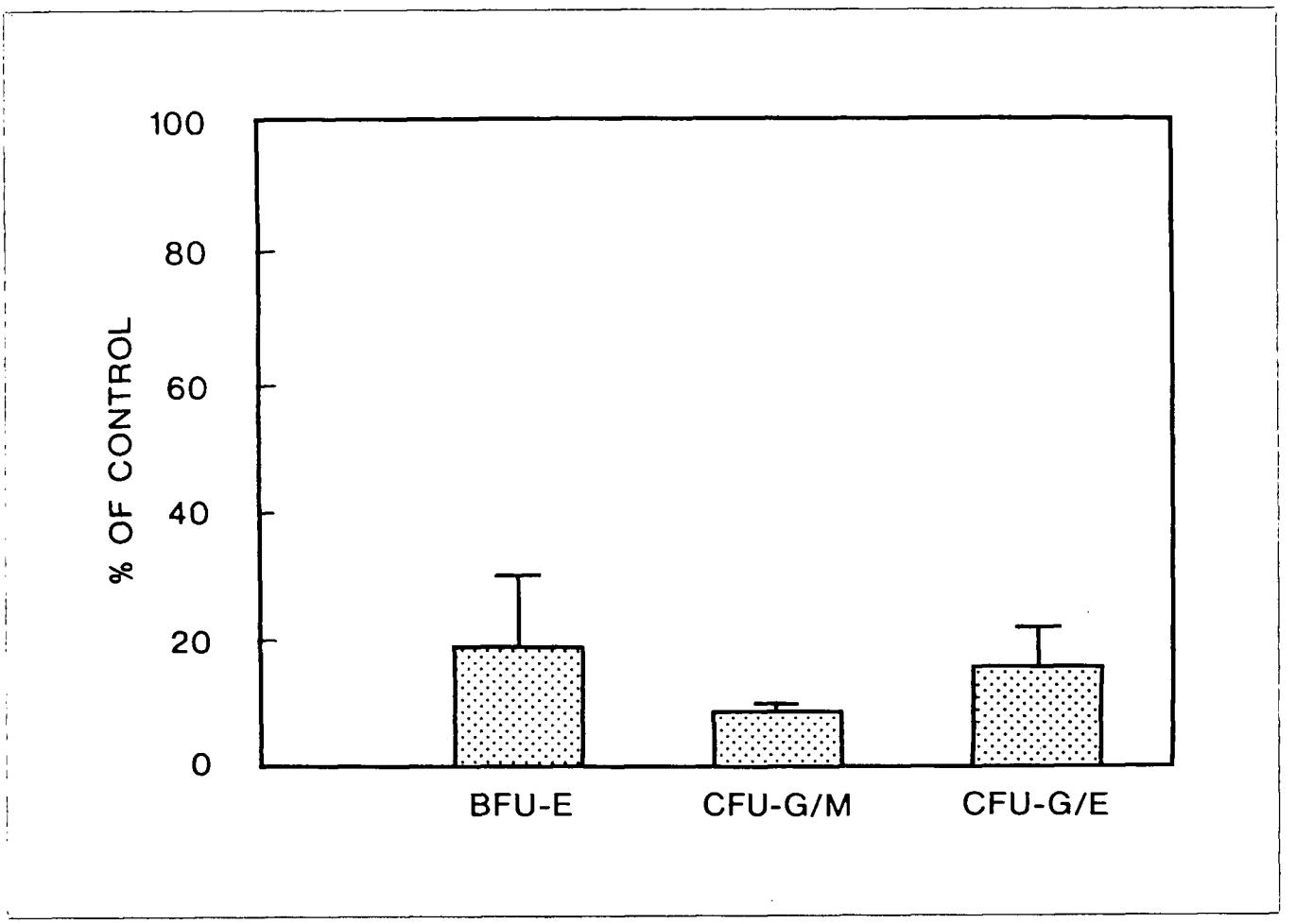

FIGURE 12. The Effects of 500 µg/ml of CHP on the Yield of Hemopoietic Progenitors in Long-Term Marrow Cultures Assessed 4-5 Weeks After Initiation. Values shown represent the mean ± 1 SEM for normalized data (% of number of progenitors from both adherent and non-adherent fractions detected in control cultures without CHP for 3 different experiments (different marrow samples). Data are from the same experiments shown in Table V.

Table VII. Lack of Effect of 500 µg/ml of CHP on Hemopoiesis in Long-Term Cultures Initiated on Pre-Established, Irradiated Adherent Layers (Feeders)

Progenitor type	Exp. no.	No. of progenitors per 10^7 cells initially seeded: + CHP + Feeder	- CHP - Feeder
CFU-G/M	1	7,324	17,792
	2	903	598
	3	7,212	1,668
BFU-E	1	1,400	768
	2	141	69
	3	808	26
CFU-GE	1	132	480
	2	24	15
	3	120	14

Values shown are the mean of 2 replicate assays from 1 culture group.

The difference between + CHP + Feeder and - CHP - Feeder is not significant ($p>0.05$ using factorial analysis of variance (26) on log transformed values).

3) DISCUSSION

A number of lines of evidence suggest that non-circulating elements of the marrow constitute an essential component of the long-term marrow culture system. In addition, they suggest that direct interactions between stromal cells and primitive hemopoietic cells may be an important part of the mechanisms which allow hemopoiesis to be maintained for many weeks in vitro in a medium to which no hemopoietic growth factors are added (4-7,21). The present studies provide additional evidence in support of this model in the human system. In a first series of experiments in which circulating myeloid progenitors were used, maintenance was present in cultures initiated in the presence of an adherent layer and to a certain extent in its absence. However, cycling data from a collaborative experiment (19) revealed profound differences between the 2 types of cultures. Primitive progenitors in the adherent layer were oscillating between proliferation (40-47% kill) and quiescence (0-18% kill) as a function of the feeding pattern. In contrast, primitive progenitors in the absence of an adherent layer were continuously cycling (44-58% kill), irrespective of the feeding schedule. However, persistence of progenitors for many weeks in the absence of a feeder layer together with the selective advantage of endogeneous lymphoblastoid cells in vitro rendered this approach impractical. Fortunately, an alternate approach could be used.

CHP, a proline analogue that is selectively toxic for collagen-producing cells, was shown to inhibit the establishment of long-term hemopoiesis by human marrow cells by virtue of its selective inhibitory action on cells that allow development of an adherent layer, at least some of which are detected by the CFU-F assay. This conclusion is based on the demonstration that the failure of hemopoiesis seen in long-term human marrow cultures initiated and maintained in

the presence of CHP at doses that inhibited the formation of an adherent layer, could be completely abrogated if a pre-established adherent layer was provided.

Both the progeny of CFU-F and a significant proportion of the fibroblast-like cells in the adherent layer are collagen-synthesizing cells (22,23). It is therefore not surprising that CHP has a particularly toxic effect on the proliferation and establishment of these cells in vitro. The fact that the same concentration of CHP did not inhibit hemopoiesis in the presence of a pre-established adherent layer suggests that collagen synthesis does not continue at high levels after the adherent layer becomes confluent or that its turnover is not crucial to hemopoiesis once a feeder layer is established. Further it indicates that such concentrations of CHP do not adversely affect the viability or regulatory function of human marrow adherent layer cells once they have become established in vitro. This confirms similar findings in the murine system (18), and suggests the usefulness of CHP supplemented medium as a way of exploiting unseparated marrow as a substitute for purified hemopoietic progenitors in future experiments designed to assess the regulatory potential of defined mesenchymal populations. This approach offers the advantage that there is no cell loss, as is usually the case during most hemopoietic cell purification procedures. In addition, other non-mesenchymal cell types, such as macrophages and T-lymphocytes, which may play a role in the long-term marrow culture system, are not eliminated from the test inoculum.

It is important to note that any procedure which seeks to provide target cells to assay for long-term hemopoietic support in the human system needs to meet the requirements of a 4-5 week endpoint. This time can be deduced from two types of experiments. The first involves the assessment of the rate of progenitor decline in long-term cultures initiated with peripheral blood cells described above in which a differential effect is seen only after several weeks

at a time when EBV-transformed lymphoblastoid cells show a selective advantage in cultures. Similarly when suspensions of marrow cells are exposed to 4-hydroperoxycyclyphosphamide and thereby selectively depleted of their content of clonogenic hemopoietic cells, but not of the more primitive hemopoietic cells from which clonogenic cells are generated in the long-term marrow culture system, regeneration of the clonogenic compartment under these conditions can be seen to require approximately 4-5 weeks (24,25). Thus any test hemopoietic cell suspension to be used to assay the hemopoietic supportive function of a candidate regulatory cell type must be able to generate clonogenic cells for at least this length of time and also show a failure of endogenous supportive function for an equivalent period. This places an additional stringent requirement on the purity of the test cells, since a very small number of stromal cell precursors over 4 to 5 weeks can reconstitute an adherent layer. In my experience this also precludes the use of non-adherent fractions of fresh or 1 week old long-term culture cells of human marrow origin for such assays. On the other hand, it appears that the use of CHP supplemented media may offer a simple and reproducible method for selectively inactivating the stromal cell component of fresh human marrow samples.

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C H A P T E R IV

INDUCIBLE PRODUCTION OF HEMOPOIETIC GROWTH FACTORS BY SV-40 IMMORTALIZED MESENCHYMAL CELL LINES OF HUMAN MARROW ORIGIN

1. INTRODUCTION

Current evidence suggests that the control of many aspects of hemopoiesis is regulated locally within the marrow (1) and may involve the activation of hemopoietic growth factor gene expression by resident non-hemopoietic cells that constitute the marrow stroma (2,3). Although some controversy exists as to the origin of these cells and their ontological relation to the hemopoietic hierarchy, studies in animal models indicate an early embryological divergence in the mesenchymal and hemopoietic components of the marrow (4). More recent experiments have established that mesenchymal cells of donor origin are not normally detectable in the marrow of transplant recipients (5,6,7) confirming earlier data in mice indicating that these cells and their precursors do not circulate (8,9). On the other hand cells with the phenotypic markers of fibroblasts, adipocytes and/or endothelial cells can be readily obtained as expanded populations in various types of human marrow culture systems. These include cultures initiated by incubating marrow cells at relatively low concentrations suitable for obtaining isolated fibroblast-like colonies (CFU-F (10), CFU-RF (6) or CFU-ST (11) assays), as well as cultures initiated with somewhat higher concentrations of marrow cells. The latter result in the formation of a complex adherent layer of mesenchymal cells that are able to support primitive human hemopoietic progenitor proliferation and differentiation for periods of many weeks (12).

More detailed studies of the cycling status of very primitive hemopoietic progenitors in long-term human marrow cultures have shown that the adherent layer plays an important role in the mechanism that regulates whether the majority of these cells are quiescent, as they are in the marrow in vivo, or whether they are proliferating in response to some perturbation (13,14). Thus for the first time, it is possible to analyze in vitro both positive and negative loops in a regulatory network that controls the behaviour of very primitive hemopoietic cells. Since long-term marrow culture adherent layers contain a variety of cells expressing different mesenchymal cell phenotypes, including 6.19-antigen-positive cells (15,16), lipid-laden adipocytes and Factor VIII-positive cells arranged in capillary-like structures (1), all of which are normal constituents of the marrow stroma, it is difficult to utilize the long-term marrow culture system to investigate the role of specific stromal components. Because all of these mesenchymal cell types are known to exhibit only a limited potential for expansion in vitro (17), novel strategies for their isolation are required. We describe the use of SV-40 for this purpose. A high titer virus stock was generated, tested for biological activity and used for the immortalization of human marrow mesenchymal cells in vitro. A large number of immortalized, cloned cell lines expressing the differentiated phenotype of fixed bone marrow stromal cells have been obtained and partially characterized. Of particular interest, is the finding that these are capable, upon stimulation, of secreting regulatory molecules that are active on primitive hemopoietic progenitors. These cell lines should be useful for the further delineation of the cellular and molecular basis of hemopoietic stem cell regulation.

2. RESULTS

(A) Transforming Potential of SV-40 Virus ans its Effect on DNA Synthesis

The transforming potential of SV-40 virus was first tested on murine NIH-3T3 cells which are known to be readily infected and transformed in vitro (18). Mock infected cultures did not show any transformed foci whereas dishes infected with SV-40 virus yielded transformed foci (Figure 13) at a frequency of 1 per $4x10^3$ cells exposed. From these experiments the transformation titer of this virus stock on NIH-3T3 was 2.7×10^6 focus forming unit/ml.

To demonstrate a direct biological effect of SV-40 that would validate its intended use in immortalizing human marrow stromal cells, we examined its effect on cellular DNA synthesis following addition of virus to contact inhibited cells subcultured from regular long-term marrow cultures adherent layers. Whereas the majority of the cells in mock infected cultures remained quiescent (Figure 14, panel A), infected cultures showed a dramatic increase in ^{3}H-Thymidine incorporation 48 hours later (Figure 14 panel B). Since these data provided an indication that SV-40 virus may be useful for the immortalization of human marrow stromal cells, this preparation was use to generate cloned human continuous lines.

(B) Derivation and Immunological Characterization of Cell Lines

Cloned lines of SV-40 immortalized cells were isolated from a variety of primary cultures. Both primary and first passage adherent layers of 3-4

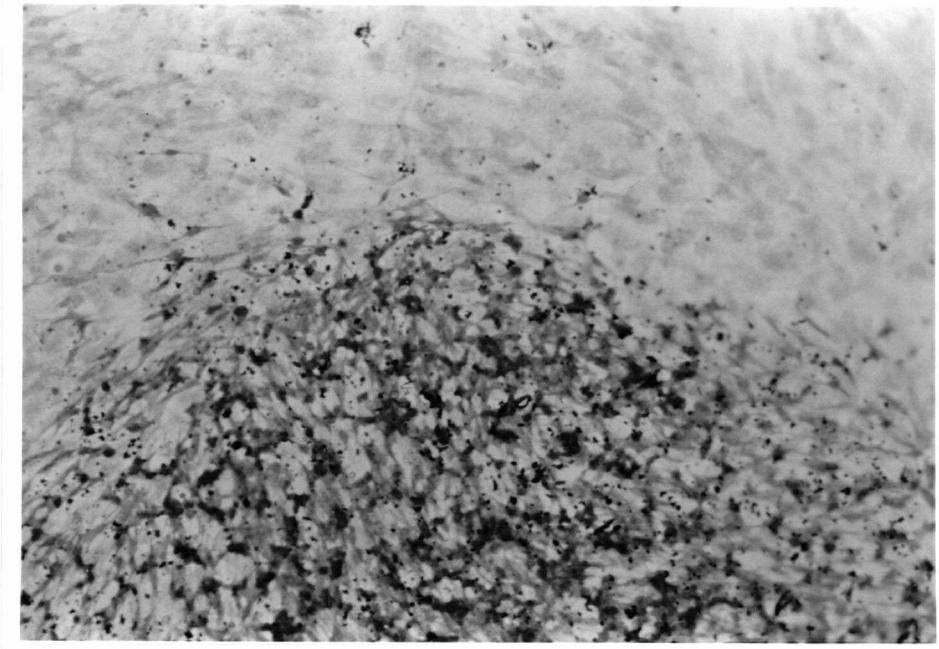

FIGURE 13. Transformed Focus of NIH-3T3 Cells.

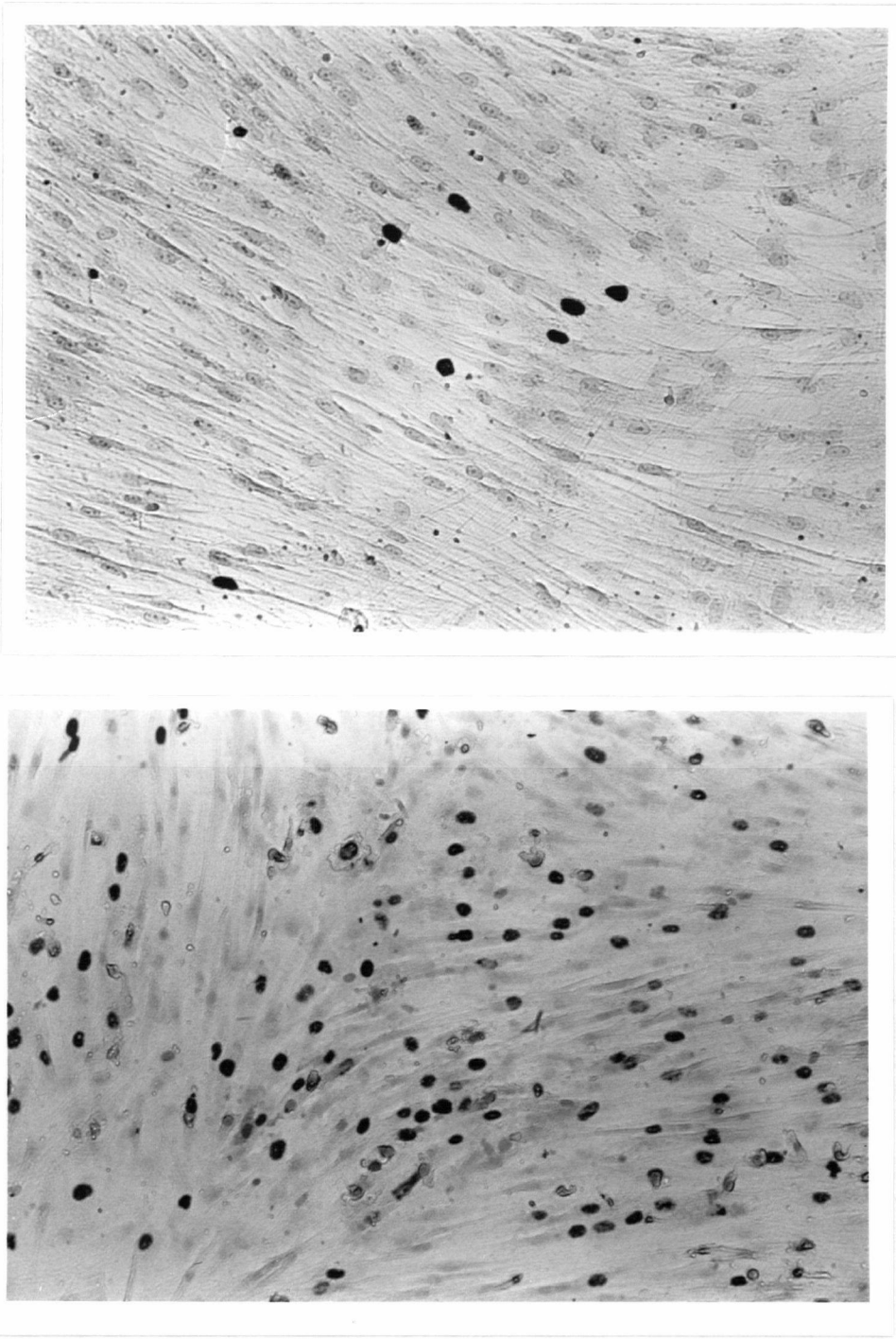

FIGURE 14. Autoradiograms of Confluent Marrow Adherent Layers. Mock infected layer (upper panel) and SV-40 infected layer (lower panel).

week old long-term human marrow cultures were used for infections since these are indistinguishable in their ability to regulate primitive hemopoietic progenitor cycling in the long-term culture system (14). Lines were also obtained following infection of "fibroblast" monolayers established from pooled colonies of human marrow origin, and HUV-EC-C endothelial cell monolayers originally derived from umbilical vein endothelium. Twenty-four hours after infection, expression of the SV-40 large T antigen was detected in 45 $\pm$ 23 % of the cells in the adherent layer of long-term marrow cultures (with higher values for infected fibroblast cultures), whereas mock-infected cells were consistently negative. Two to 3 weeks after infection, low density subcultures could be seen to contain foci of morphologically altered cells at a frequency of ~1 focus per 100 initially T Ag-positive marrow cells. The cells in these foci were more elongated and refractile than normal and grew in a disordered fashion typical of cells whose growth is not contact inhibited. This morphology was exhibited by all transformed foci and was independent of the type of population initially exposed to the virus. Lines derived by infecting long-term marrow adherent layers are designated as MH, lines derived from marrow fibroblasts as CFUST, and lines derived from endothelial cells as EC. From these three types of cells, a total of 13, 13, and 5 such lines were independently isolated. After cloning, each line was expanded and then frozen in DMSO and stored at -70°C until required for further study. Many lines have been maintained continuously for periods of months and, in one case, (CFUST-16) for over a year.

The results of immunophenotyping studies are summarized in Table VIII. All cells in all lines were positive for the SV-40 large T antigen indicating a functional integrated SV-40 genome consistent with their immortalized state. All lines tested (5/5) were also positive for the surface marker 6.19

Table VIII. Histochemical and Immunophenotypic Properties of SV-40 Transformed Cell Lines*

Marker Assessed	Line Origin: Long-Term BM Adherent Layer Cells (MH2SV-C11)	BM Fibroblast Colony Cells (CFUST-C116)	Umbilical Cord Endothelial Cells (EC22)
Membrane			
6.19	+	+	+
T200	-	-	+
LEU-M1	-	-	-
LEU-M3	-	-	-
Cytoplasmic			
Acid Phosphatase	+	+	-
Alkaline Phosphatase	-	-	-
Factor VIII	-	-	+
Collagen I	+	+	-
Collagen IV	+	+	+
Laminin	+	+	+
Nuclear			
SV-40 large T Ag	+	+	+

*Data shown are for representative lines, for which the most complete documentation was obtained. Positive means that more than 30% of the cells were positive. Negative means no positive cells could be detected.

(Figure 15) which appears to react only with mesechymal cells in the marrow (15,16). Marrow-derived lines were phenotypically similar, regardless of the type of culture initially infected and were consistently positive for acid phosphatase, laminin and collagens type I and IV. A significant proportion of cells in the adherent layer of long-term human marrow cultures also show these features as do cells produced by CFU-RF (6)). Although I showed that cells positive for Factor VIII-related antigen can also be found in the adherent layer of long-term marrow cultures (1), none of the marrow-derived transformants showed this property. This could not, however, be attributed to an inability of SV-40 to immortalize Factor VIII-positive cells as shown by the continued expression of this antigen in lines derived following infection of HUV-EC-C cells. None of the lines, including representatives from all three categories (by origin) contained detectable LeuM1 or LeuM3 positive cells. Marrow-derived lines were also consistently negative for T200 (Figure 15). However, SV-40 transformation of HUV-EC-C cells did activate weak T200 positivity (in the one line tested), whereas untreated HUV-EC-C cells were consistently negative (Figure 16).

(C) Characteristics of Transformed cell lines

The SV-40 large T antigen, a multifunctional 90-100 Kd oncogenic protein, is expressed in all SV-40 transformed cells (19). In addition to its role in virus replication, it exerts numerous effects on susceptible host cells including the stimulation of cellular DNA synthesis even in quiescent cells (20). A longer lasting effect of SV-40 on these same cells has also now been documented. This can be seen as a decreased population doubling time

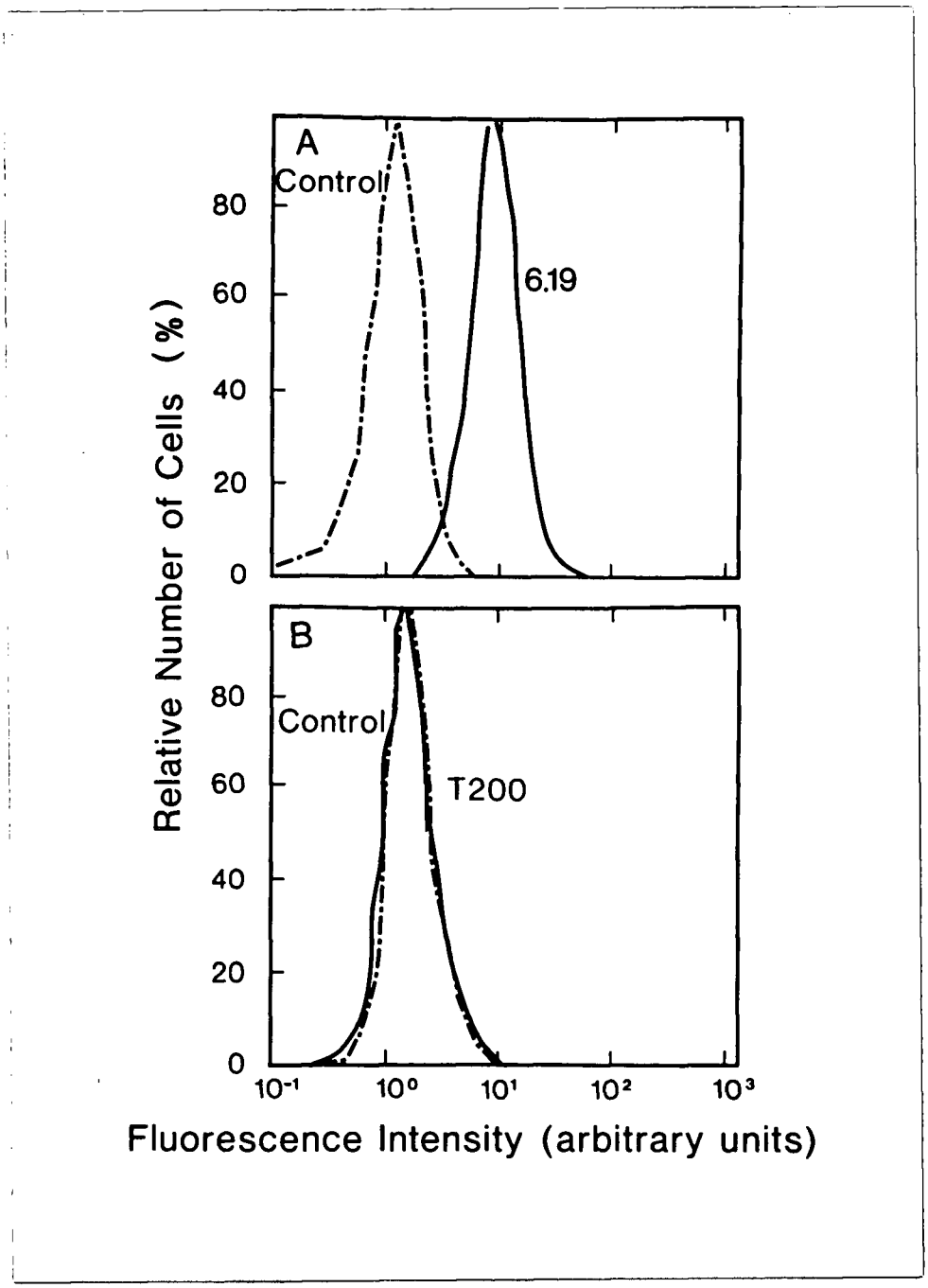

FIGURE 15. FACS Profile of CFUST-CL16 Cells Stained With Monoclonal Antibody 6.19 (Panel A) and Anti-Leuk/T200 (Panel B). The solid line shows the profile of the test sample in each case. The dotted line shows the profile of the corresponding negative control sample.

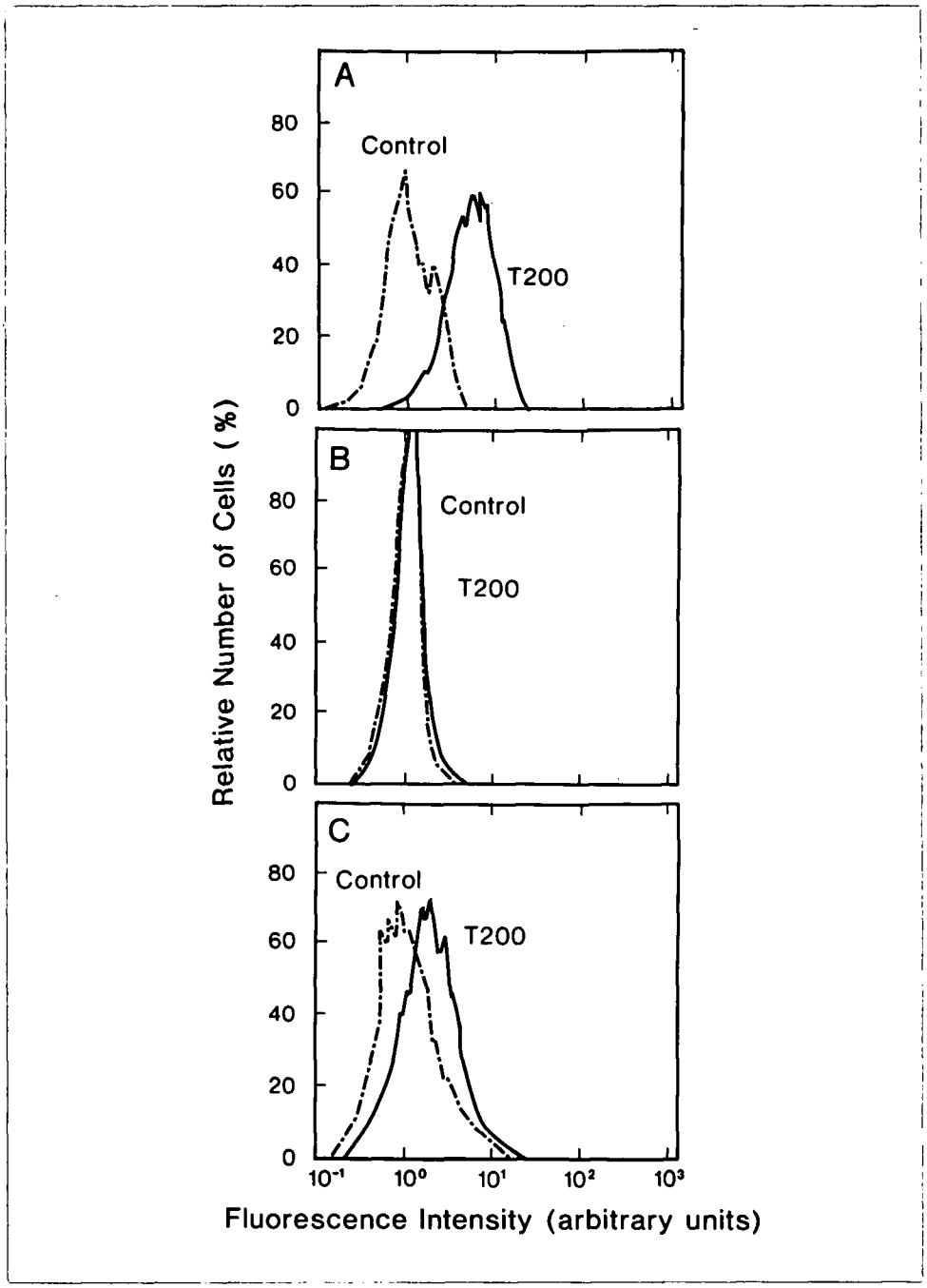

FIGURE 16. FACS Profile of a Suspension of Spontaneously Immortalized Lymphoblastoid Cells (Panel A), and HUVE-EC-E Cells Before (Panel B), and After (Panel C) Transformation With SV-40 Virus.

(Figure 17 B), or an increase in ^{3}H-thymidine incorporation (Figure 17 A) of infected as compared to non-infected cells.

Capacity for anchorage-independent growth was tested by plating transformed and uninfected cells in semi-solid medium. SV-40 transformed lines routinely formed distinct colonies containing more than 100 cells within 20 days in culture (Figure 18 A). In contrast, cells from non-infected control cultures yielded only a few small clusters of 4 to 8 cells and did not form any colonies containing more than 50 cells (Figure 18 B). After cloning, the plating efficiency of the 3 lines tested remained high (>5%) and was independent of cell concentration (Figure 19), although some variability in the colony-forming efficiency of different lines was seen (data not shown).

(D) Induction of Growth Factor Production

Recent reports have shown that fibroblasts and endothelial cells of various tissue origins constitutively produce low or undetectable levels of hemopoietic growth factors, but upon stimulation with various secretory products of macrophages, including IL-1, hemopoietic growth factor production is rapidly and markedly enhanced (2,3,21,22). This response thus appears to be a tightly regulated part of the functional program of a variety of mesenchymal cell populations. Recent data from the Terry Fox Lab have shown that IL-1β also stimulates the production of hemopoietic growth factor production by cells in the adherent layer of long-term human marrow cultures (23). It was therefore of interest to evaluate the activity of media conditioned by the various transformed cell lines before and after exposure to IL-1β. The results of two representative experiments are shown in Table IX. It can be seen that addition of 12 units/ml IL-1β greatly enhanced the production and release of

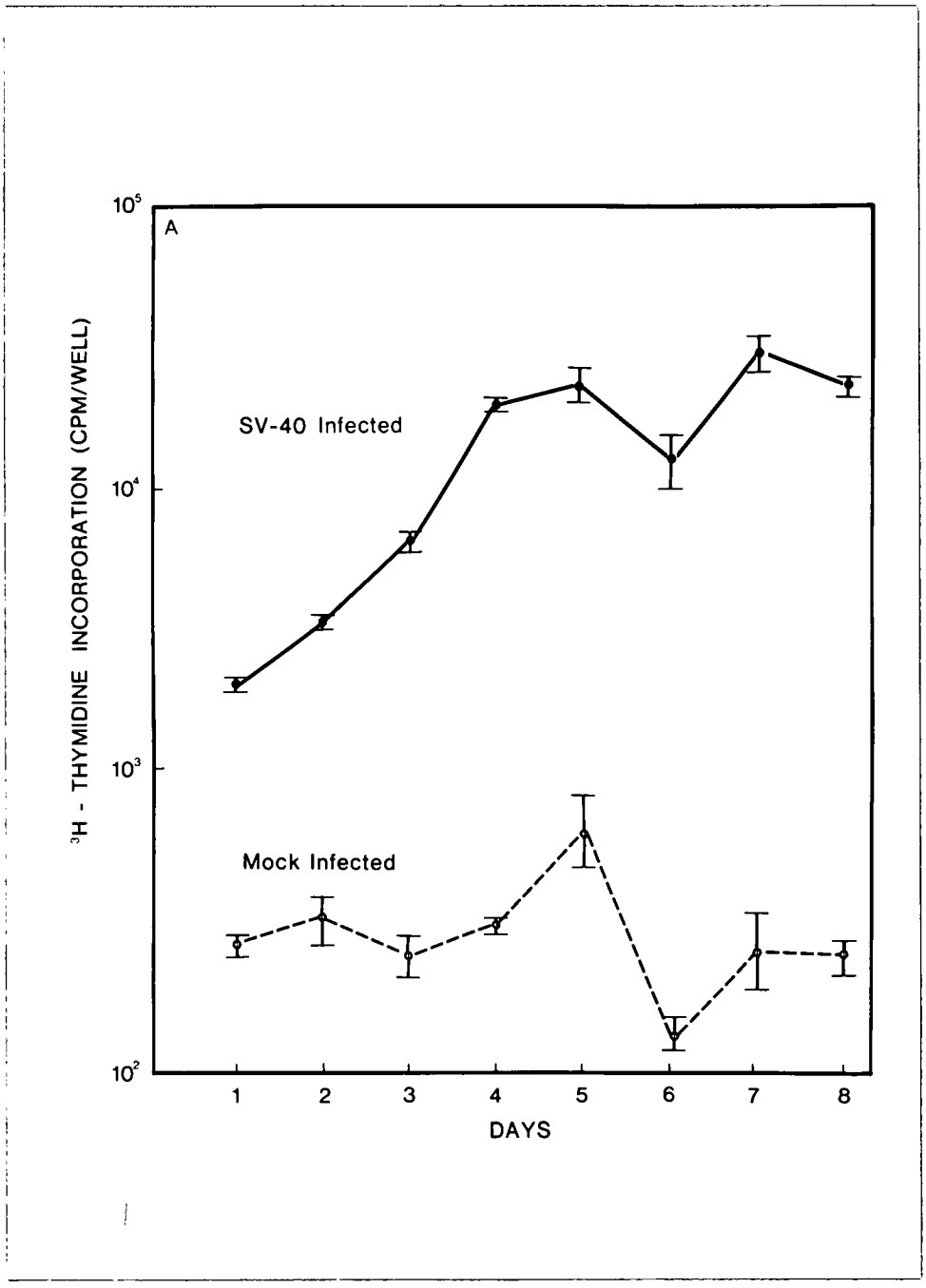

FIGURE 17. Tritiated-Thymidine Uptake (Panel A) of SV-40 Infected (Solid Lines) and Uninfected (Broken Lines) MH Cells.

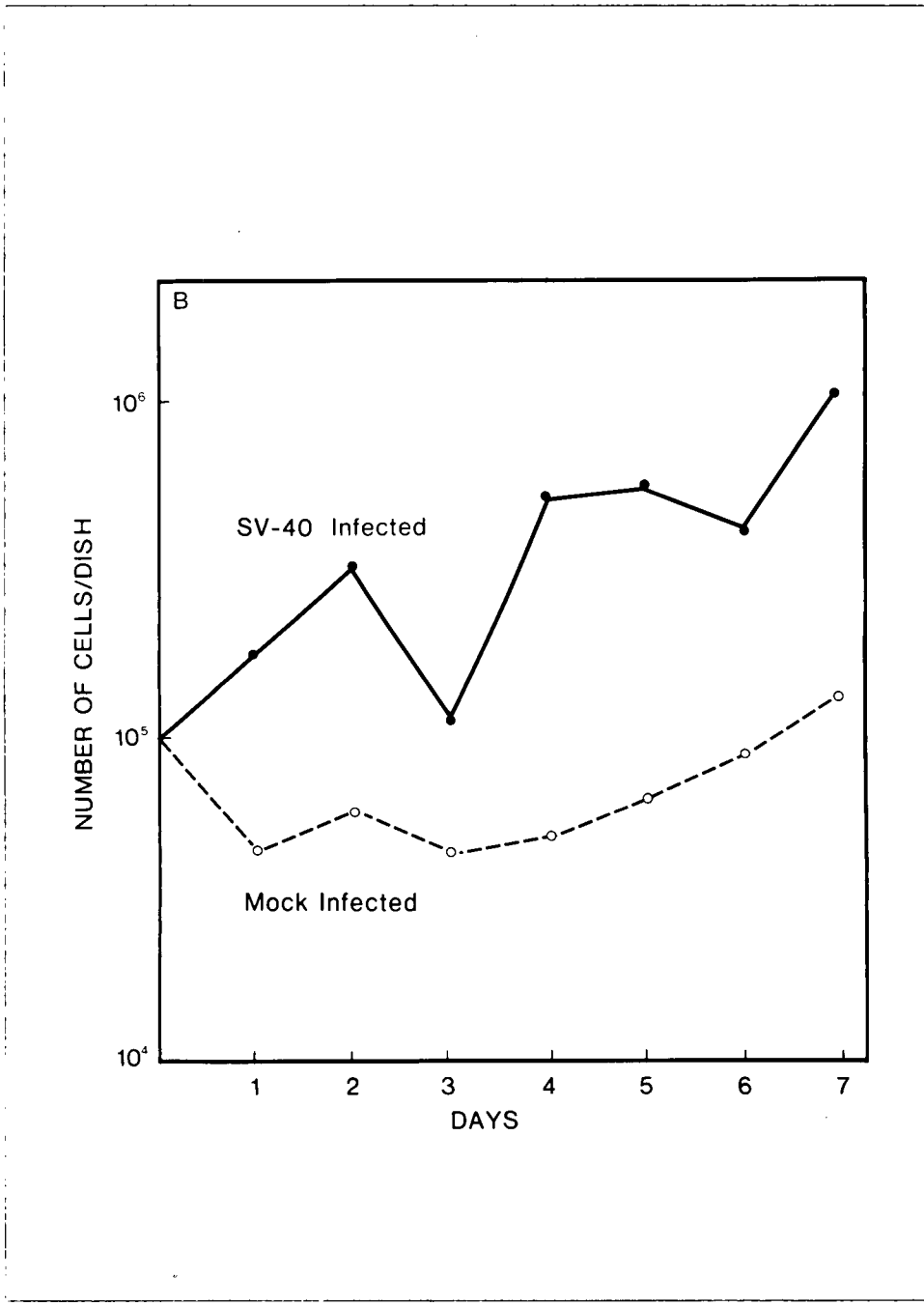

FIGURE 17. Growth Rate (Panel B) of SV-40 Infected (Solid Lines) and Uninfected (Broken Lines) MH Cells.

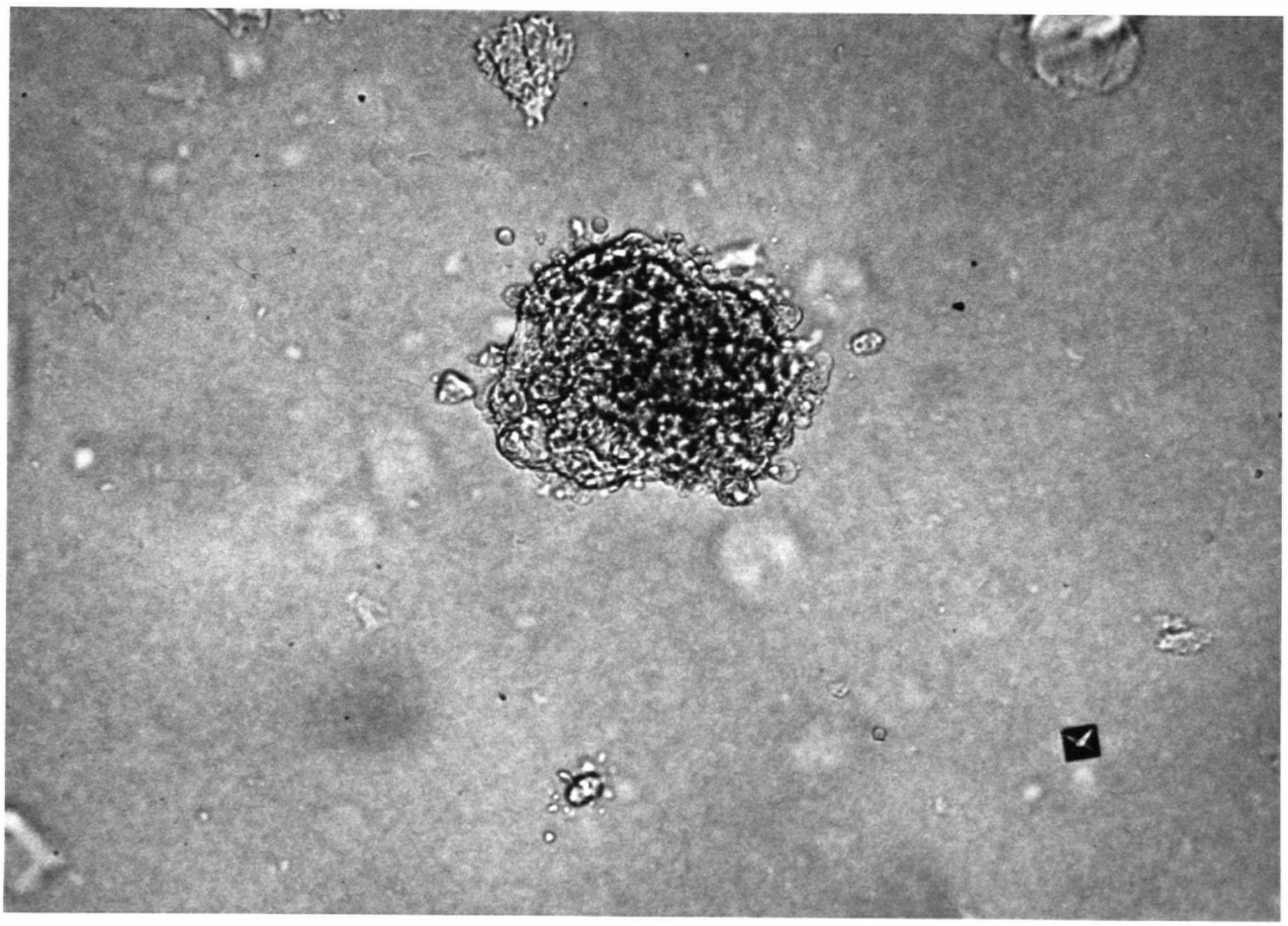

FIGURE 18. A Colony of Transformed Cells Generated in a Methylcellulose Culture 14 Days After Seeding the Cultures with MH Cells Infected With SV-40 Virus (Panel A).

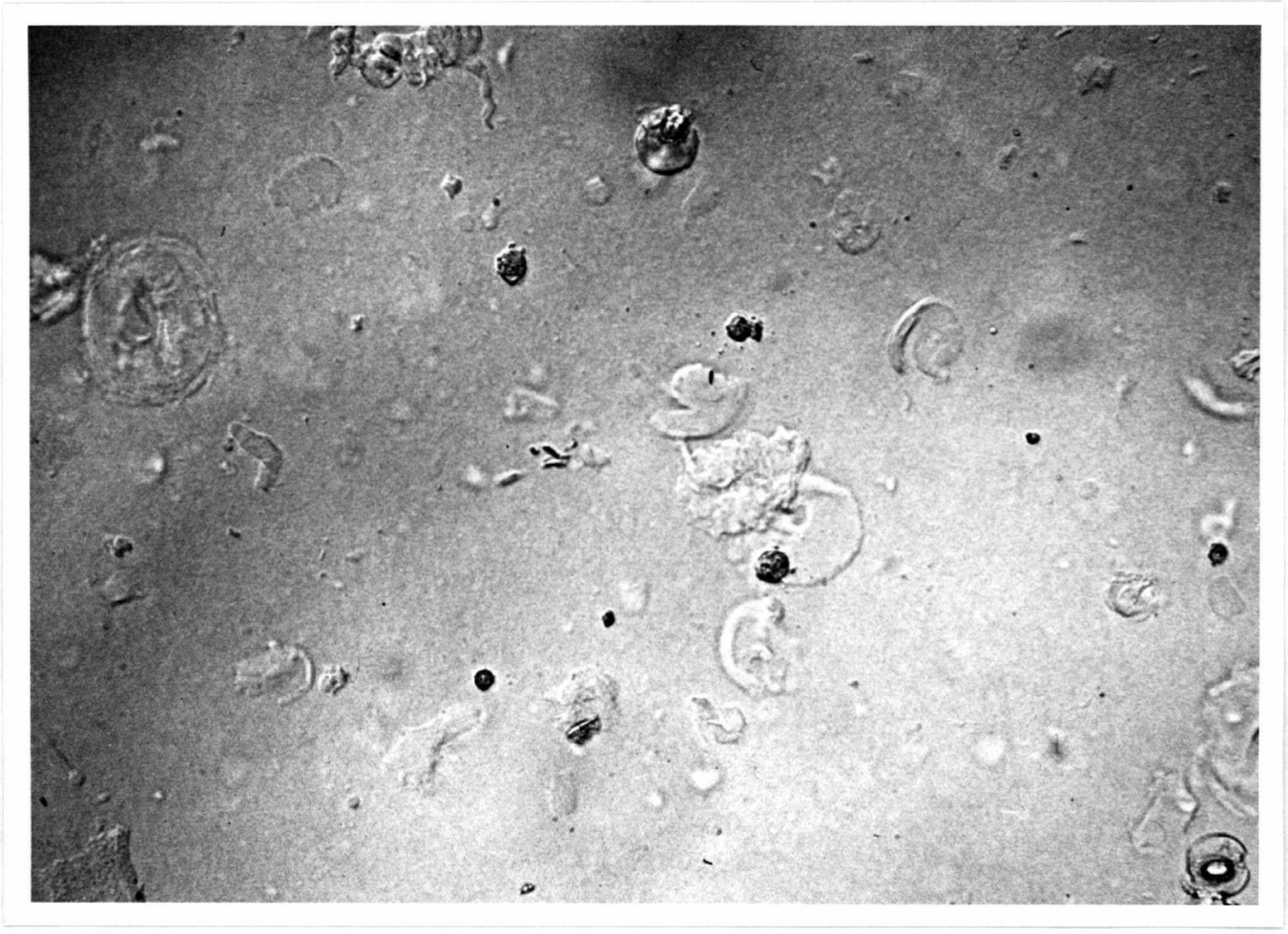

FIGURE 18. Control (Uninfected) MH Cells Failed To Yield Colonies (Panel B).

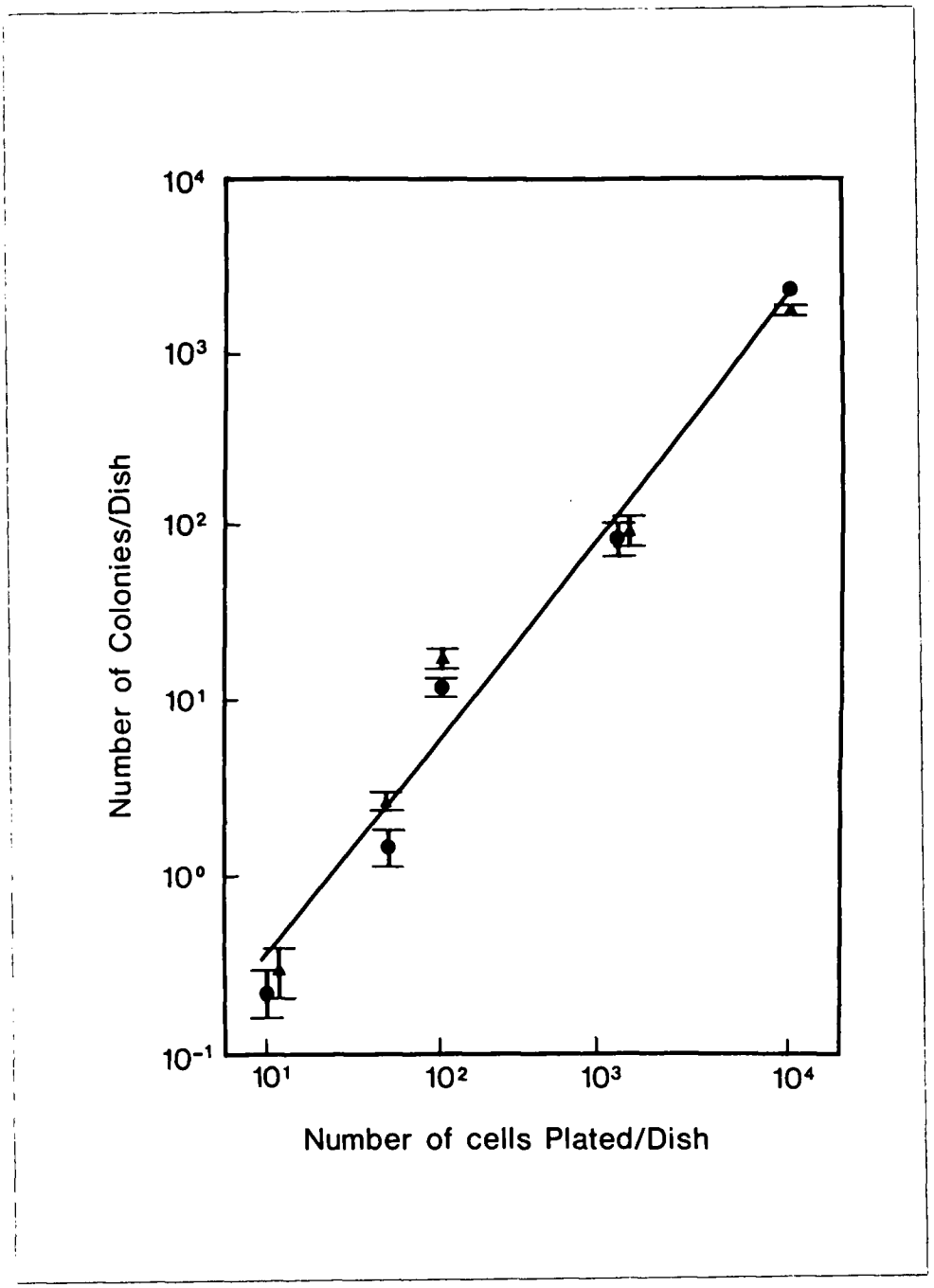

FIGURE 19. Analysis of the Clonogenic Capacity of MH2SV-CL1 Cells Plated in Methylcellulose. Each data point represents the mean ± 1 SEM of values obtained in each of two different experiments (shown separately as circles and triangles).

Table IX. Evidence for IL-1β Induced Production of Hemopoietic Colony-Stimulating Activity by Representative SV-40 Transformed Human Cell Lines

Addition to Methylcellulose Assay	Erythroid Colonies		Granulocyte-Macrophage Colonies	
	Exp 1	Exp 2	Exp 1	Exp 2
MH2SV-C11	1	3	17	19
MH2SV-C11 + IL-1β	7	7	111	124
CFUST-C116	3	2	32	8
CFUST-C116 + IL-1β	8	4	108	99
EC22	0	2	9	2
EC22 + IL-1β	9	4	97	26
Skin Fibroblasts	1	-	6	-
Skin Fibroblasts + IL-1β	2	-	56	-
No addition	0	0	0	0
IL-1β (1.2 units/ml)	3	4	47	23
M2-10B4 (mouse marrow fibroblast line)	-	3	-	12
M2-10B4 (mouse marrow fibroblast line) + IL-1β	-	3	-	25
Human LCM (10%)	18	9	126	115
GM-CSF (8 ng/ml)	5	3	168	86

*Cells were incubated with or without 12 units/ml of IL-1β for 24 hours as described in the Materials and Methods. Conditioned media were added to methylcellulose assays at a final concentration of 10% (v/v). Erythroid colonies include all categories (CFU-E plus BFU-E derived). M2-10B4 cells are a spontaneously immortalized cloned line of cells phenotypically similar to the MH and CFUST lines described here but derived in this laboratory from a culture of adherent mouse marrow cells.

colony-stimulating factor(s) active on primitive human hemopoietic progenitor classes on two separate differentiation lineages. A total of 15 lines were tested in this way. All showed this response. Although there was some variation in the final activity of the conditioned media from different lines exposed to IL-1β, an activity equivalent to or greater than 80 ng/ml of GM-CSF was often obtained. In contrast, similarly prepared conditioned media obtained from IL-1β stimulated marrow stromal lines of murine origin had no activity on human progenitors (Table IX). This suggests that the weak activity sometimes seen in controls given IL-1β alone was due to endogenous production of colony-stimulating activity by co-existing cells in the methylcellulose assay and that residual IL-1β concentrations in the test conditioned media were considerably reduced (below 1.2 units/ml). Dose response studies established that 12 units of IL-1β/ml was well above the minimal dose required to attain maximal growth factor release (Figure 20). Conditioned media were also assayed (Dr. P. Lansdorp, Terry Fox Laboratory) for IL-6 activity as measured by their ability to stimulate ^{3}H-thymidine incorporation into B13.29 cells, a murine hybridoma cell line that is specifically responsive to IL-6 (24). Results from these studies have indicated that the production of IL-6 bioactivity by the SV-40 transformed lines, like the production of colony-stimulating bioactivity was consistently found to be markedly increased by the stimulation of the lines with IL-1β.

Since there is now evidence that hemopoietic growth factors may act not only on hemopoietic cells but also on target populations of widely diverse embryonic origin (25), I also investigated the possibility that medium conditioned by SV-40 marrow immortalized lines might have biological activity on normal mesenchymal cells. As shown in Table X, conditioned media from one

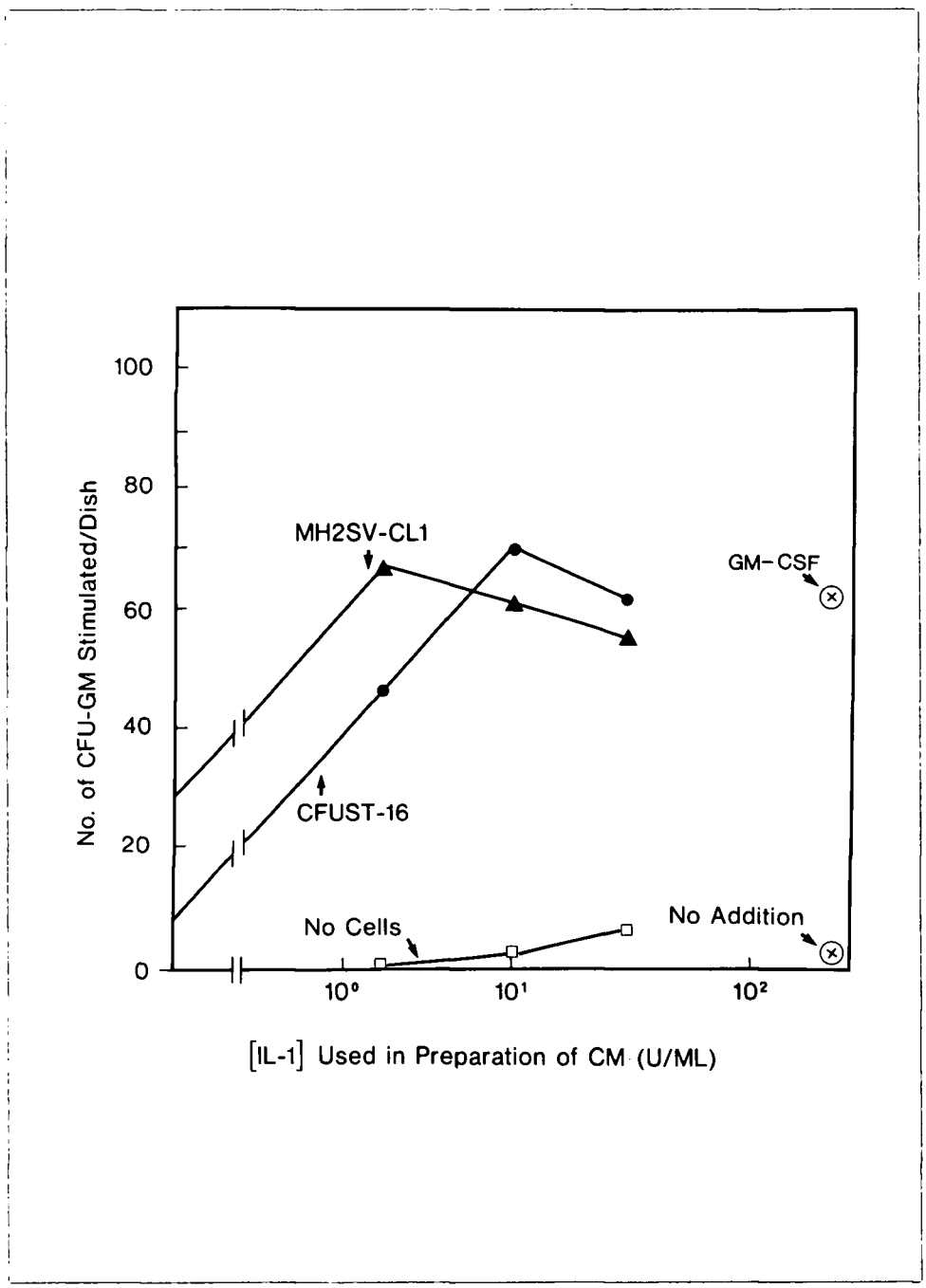

FIGURE 20. Hemopoietic Colony-Stimulating Activity of Media Conditioned for 24 Hours by CFUST-CL16 or MH2SV-CL1 Cells (or No Cells) as a Function of the Concentration of IL-1β Used as a Stimulant. The maximal number of colonies obtainable from the progenitors present in the assay as indicated by stimulation of these with an optimal concentration of recombinant human GM-CSF (8 ng/ml) is also shown for comparison. Cell line conditioned media were present in the methylcellulose assays at a concentration of 10% (v/v).

Table X. Effects of Conditioned Medium of an IL-1 Stimulated, SV-40 Immortalized Marrow Stromal Cell Line (CFUST-16) on CFU-F Formation

Addition (final concentration in CFU-F assay)	No. of CFU-F/Dish			
	Exp. 1	Exp. 2		Exp. 3
	(Day 12)[1]	(Day 5)	(Day 10)	(Day 6)
Medium (α-10%)	13	10	58	8
Human PHA Stimulated Leukocyte CM (10%)	29	24	62	9
CFUST-16 with IL-1 CM (10%)[2]	28	24	95	14
CFUST-16 without IL-1 CM (10%)	-	-	-	7
GM-CSF (8 ng/ml)	29	22	100	24

[1]No. of days of CFU-F colony growth prior to fixation and staining.

[2]Conditioned medium from confluent CFU-STCl16 cell monolayers incubated for 24 hours with or without 12 U/ml recombinant human IL-1β (Biogen).

line tested (CFUST-16) shared with GM-CSF the ability to stimulate the plating efficiency of fresh normal bone marrow fibroblasts.

(E) Irradiation studies

To examine the possibility that SV-40 could transform human marrow stromal cells without altering their radiobiological properties, cell survival curves were obtained from normal and SV-40 transformed lines after single dose irradiation. In both cases, survival curves are exponential and shouldered with a D_0 of 1.3 Gy (Figure 21) which is in accord with previously reported data on cultured bone marrow fibroblasts (26).

3. DISCUSSION

In humans, hemopoiesis is normally restricted to the bone marrow where it proceeds in close association with as yet poorly characterized fixed mesenchymal elements. Recently, an vitro model has been developed in which the long-term maintenance and regulated turnover of primitive hemopoietic progenitors occurs in the absence of exogenously provided growth factors. However, a key component of this culture system is a complex adherent cell layer containing a variety of mesenchymal cell types of marrow origin. To facilitate analysis of the individual roles of these various mesenchymal cell types, I used SV-40 virus as an immortalizing agent. A high titer virus stock was prepared and its biological activity tested on both murine and human mesenchymal cells. Lines could be readily obtained from human long-term marrow culture adherent layers, and marrow fibroblast cultures. Both of these contain cells with properties similar to those exhibited by all marrow-derived cell

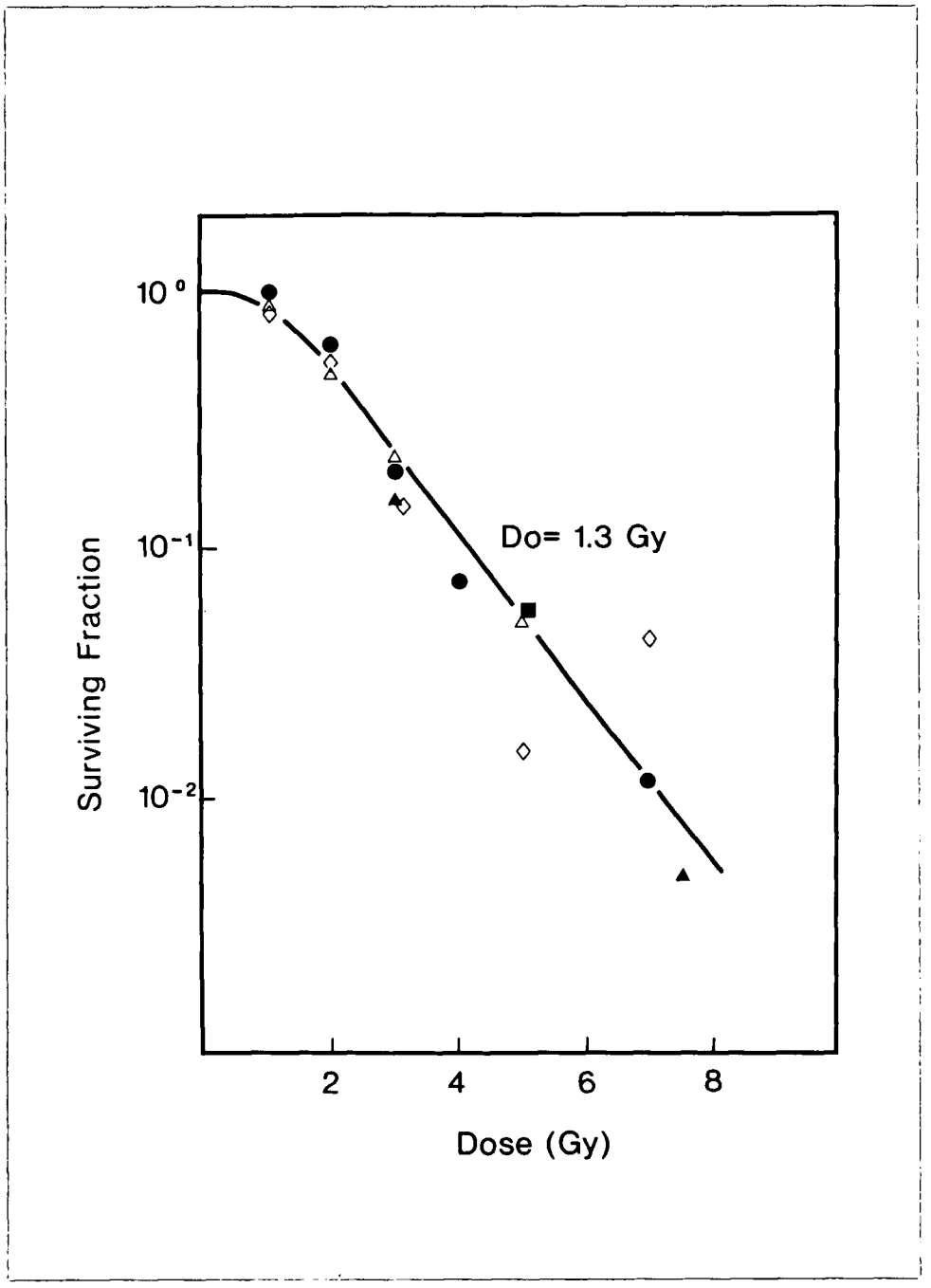

FIGURE 21. Cell Survival Curve for SV-40 Immortalized Marrow Fibroblasts. CFUST-CL16 (open and solid triangles), SV-40 immortalized endothelial cells EC CL22 (open diamond), Factor VIII positive cell hybrid Ea.926 (solid circles), and normal bone marrow fibroblasts (solid squares).

lines studied. This phenotype is characterized by the expression at the surface of the antigen 6.19 and in the cytoplasm of laminin and collagen IV, with a lack of expression of Factor VIII. Two other reports of SV-40 immortalized human marrow cells have been published. In one, phenotypic characterization suggested a fibroblastic origin of the line (27). In the other, the presence of "round" cells that reacted both with the pan-hemopoietic cell determinant, T200, and the monocytic antigen, LeuM3 was a consistent finding (28). In contrast, I did not observe a subpopulation of more spherical cells in any of my lines and a search for evidence of LeuM3 or T200 expression gave consistently negative results with one exception. This was the expression of detectable T200 surface antigen after (but not before) transformation of HUV-EC-C endothelial cells with SV-40 suggesting that SV-40 may cause some phenotypic alteration of mesenchymal cell antigen expression when used as a transforming agent. This was not surprising since preliminary data have indicated that SV-40 transformed marrow cells were also grossly abnormal cytogenetically. On the other hand, I have found in a collaborative study that SV-40 transformed cells are stimulated by GM-CSF (29) as was also noted by Singer et al (28). As mentioned earlier such responsiveness has further been demonstrated to be a feature of a variety of developmentally unrelated cells of non-hemopoietic origin (25). GM-CSF responsiveness can therefore not be used to indicate a close relationship to hemopoietic cells.

SV-40 transformed cells grew at a faster rate than non-infected cells and showed other properties of transformed cells such as loss of contact inhibition and the acquisition of anchorage-independence. Interestingly, this could not be correlated with tumorigenicity since at least one line when injected into 4 nude mice failed to generate any tumors (data not shown), a finding also reported by others for SV-40 transformed cells (30). This could not be

correlated either with increased radiosensitivity since both SV-infected and non-infected cells had similar response to ionizing radiation.

The ability of transformants to form colonies in semi-solid medium, particularly at very low cell concentrations was consistent with the likely single cell origin of the colonies obtained under these conditions. This was confirmed by assessment of a methylation sensitive restriction fragment length polymorphism in the X-linked HPRT gene (31) in the DNA of a clone isolated from a female heterozygote (data not shown, obtained by Dr. Ali Turhan, Terry Fox Laboratory). Thus, these studies have demonstrated the feasibility of generating a large number of clonal lines and subclones by exploiting their ability to form colonies in semi-solid medium.

In addition, our results indicate that upon stimulation with as little as 3 units/ml of IL-1β, all SV-40 transformed lines displayed the ability of normal mesenchymal cell populations to show a marked induction of hemopoietic colony-stimulating factor production, including the production of at least GM-CSF and IL-6. This has been confirmed at the molecular level by Northern Blotting and S1 mapping analyses (Dr. RK. Humphries, Dr. R. Kay, Terry Fox Laboratory). Recently, it was demonstrated that primitive progenitors in the adherent layer of long-term marrow cultures can be activated into cycle following the addition of IL-1β (32). In addition, data suggesting that IL-6 may synergize with other colony-stimulating factors to stimulate the proliferation of very primitive hemopoietic cells in methylcellulose has been reported (33). Lastly, the finding that medium conditioned by IL-1β stimulated SV-40 transformed lines stimulates the clonal growth of fresh normal fibroblastic cells is intriguing. It raises the tantalizing possibility that inflammatory cells, monocytes for instance, may recruit mesenchymal cells locally to produce hemopoietic growth factors which could in turn participate

in various physiopathologic processes including wound healing, atherosclerosis, myelofibrosis and possibly desmoplastic reaction of tumors. The availability of permanent cloned lines of cells with a stromal phenotype that exhibit the regulated ability to produce such factors should facilitate future studies of the role that these cells may play in the control of hemopoietic stem cell turnover.

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CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

In humans, hemopoiesis is maintained for the lifetime of the individual through the differentiative divisions of a limited number of hemopoietic stem cells (1). These cells originate in the yolk sac mesoderm and then undergo a series of migrations early in ontogeny to eventually establish themselves in the extravascular spaces of the bone marrow of the adult. There they are found in close proximity to a number of fixed bone marrow elements which collectively constitute a hemopoietic "microenvironment" (2).

Very little is known about the molecular events involved in the commitment of pluripotent hemopoietic stem cells. However, over the past several years evidence has accumulated to support the idea that the proliferative state of hemopoietic stem cells is extrinsically regulated through short-range interactions with non-circulating bone marrow cells (3). While the exact nature of the signals involved in these interactions awaits full biochemical characterization, an attractive hypothesis is that marrow stromal cells respond to perturbations affecting mature blood cells which results in the local secretion of hormone-like hemopoietic growth factors or the expression by stromal cells of surface bound forms of such growth factors.

Primitive hemopoietic cells cannot be recognized morphologically. Studies on the earliest proliferative events occurring in hemopoietic differentiation have therefore come to rely mainly on in vitro culture techniques that detect these cells by measuring their developmental potential under defined conditions. In vitro colony assays have been invaluable in providing a conceptual framework about the hierarchical organization of the

hemopoietic system. They have also led to the initial discovery and identification of a number of distinct hemopoietic growth factors (4). However, colony assays have certain intrinsic limitations. First, they only support progenitor cell limited self-renewal. Second, by their very nature, they minimize cellular interactions with stromal cells which are suspected to play a critical role in the regulation of stem cells in vivo (5). Thus, the description by Dexter et al. (6) of an alternative in vitro system which allows the long-term maintenance of high proliferative potential progenitor cells was nothing less than revolutionary. For the first time, it became possible to study the turnover of hemopoietic stem cells under in vitro conditions which allow, and likely require, close interactions between stem cells and marrow stromal cell types. Utilization of the long-term marrow culture system by many different groups has since provided considerable evidence that this is the case. However, many important questions are still unanswered. For example, the nature of the cellular regulatory signals that regulate stem cell turnover in this system has not yet been established nor has their origin.

These issues cannot be resolved without first dissecting out the individual components of the long-term marrow culture system and then developing experimental approaches to reconstruct the function provided by the adherent layer. The purpose of my research was to initiate studies along these lines, based on the assumption that the mesenchymal cells of the marrow are important in the regulation of hemopoiesis. To achieve this goal, I set myself the task of isolating and then analyzing these elements from the long-term marrow culture system.

The first goal was to develop an assay that would allow quantitation of the hemopoietic supportive function of long-term culture adherent layers which

might then later be applied to assess the essential cellular components within these cultures once such individual cell populations had been isolated. Ideally, such an assay had to be reproducible and easily set-up using commonly available sources of hemopoietic cells (e.g. regular marrow aspirates or peripheral blood). Thus, I wished to avoid or minimize the use of lengthy and expensive cell separation procedures if possible. Since contamination by mesenchymal cells would markedly reduce the sensitivity of the assay, selection of a suitable target population was the major concern.

Results of cocultivation experiments initiated with T-depleted light density peripheral blood cells indicated that progenitors from that source could be maintained on pre-established adherent layers for at least 3 weeks. This finding was exploited in a collaborative study to analyze the proliferative status of progenitors maintained in the presence or absence of an adherent layer. These experiments revealed that primitive progenitors were down regulated only when close interactions with the adherent layer were maintained and that peripheral blood itself did not contain cells able to establish an adherent layer (7). These results validated the feasibility and usefulness of reconstitution experiments to study the regulation of hemopoiesis by non-hemopoietic stromal cells. However, they also revealed that this strategy was impractical for routine longterm assays of hemopoietic supporting functions.

I therefore next turned my attention to bone marrow as an alternative source of hemopoietic progenitor cells. However, because bone marrow aspirates contain mesenchymal progenitor cells it was necessary to develop a strategy to adequately eliminate or suppress these cells to reduce the background in control dishes to an acceptable level. Although many investigators have described potential methods for this (8,9,10,11), most

proved unsatisfactory. One which did work however was based on experimental evidence in the murine system which suggested that Cis-4-Hydroxy-L-Proline (CHP) could be used to selectively inhibit stromal cell proliferation. CHP is a relatively specific inhibitor of collagen synthesis. When I tested its effects on the stromal and hemopoietic components of normal human bone marrow a significantly differential effect on these two cell types was revealed. These studies showed that CHP could be used to completely block marrow mesenchymal cell proliferation at doses that had no effect on the stem cell regulatory functions of such cells once they had already been established as a confluent layer in vitro. These findings provided further evidence that collagen-producing mesenchymal cells of marrow origin play an important role in promoting hemopoiesis in long-term marrow cultures. Moreover they showed that supplementation of long-term marrow culture medium with CHP makes it possible to use unseparated marrow cell suspensions to investigate and define mesenchymal cell types that may have stem cell regulatory properties.

To further characterize the cell populations present in long-term marrow cultures and their respective functions as part of the hemopoietic environment, a major effort was then directed at obtaining cloned lines of individual marrow stromal cells. Since human mesenchymal cells senesce in vitro, some immortalization procedure was needed. Recently, several groups have reported that SV-40 virus can be used to transform various human cells type without alteration of the original differentiated phenotype of the cells. I therefore set out to generate a high titer preparation of SV-40 and then use it to isolate a large number of a continuous cell lines of human marrow and umbilical cord endothelial cell origin.

Lines were derived from long-term culture adherent layers and marrow fibroblasts. These all expressed the same phenotype as cells present in the

non-infected population. All were positive for collagen I, collagen IV, and laminin, but negative for factor VIII. None expressed any of the differentiation markers of hemopoietic cells that were examined. Several immortalized endothelial lines were also obtained. These too resembled the parent cells phenotypically, with the one exception that weak activation of T200 expression was noted in one line examined. However, all of these lines did exhibit altered properties that are generally characteristic of transformed cells. These include a reduced doubling time, ability to grow to a higher cell density, and anchorage independence. This latter property was sometimes exploited to clone the lines.

A major conclusion from the first functional studies performed with these lines was that they retained the ability of normal parent mesenchymal cells present in long-term marrow cultures to show a marked production of hemopoietic colony stimulating factors upon induction with IL-1. Additional information about the probable identities of the factors produced was obtained in a collaborative study with Dr R.K. Humphries and Dr R. Kay who demonstrated a substantial increase in GM-CSF and IL-6 mRNA levels following induction using Northern blotting and S-1 protection analysis techniques. The presence of IL-6 itself was further confirmed by specific bioassays using an IL-6 dependent cell line.

The enhanced production of readily detectable levels of hemopoietic growth factors such as GM-CSF and IL-6, both of which can act on primitive hemopoietic cells (12) is a tantalizing finding. However, I have presented evidence that regulation of primitive hemopoietic cells is likely to operate at the local level rather than via the circulation of released factors. Obviously, one possibility is that the localization of responses apparent in vivo is simply due to the dilution of released growth factors to ineffectual

levels at more distant regions. Recently, evidence was presented to indicate that GM-CSF can also bind to glycosaminoglycans of the marrow extracellular matrix. This suggests an intriguing mechanism by which released growth factors might be locally concentrated in tissues. Another possibility is that enhanced production of secreted forms of growth factors might be accompanied by a biologically more important enhanced production of membrane-bound growth factor molecules. Such a possibility has a precedent in the case of M-CSF (13). However, these findings do not address the question of how negative regulation of primitive hemopoietic cells is achieved either in vitro or in vivo. One possibility is that negative regulation occurs at the level of the regulatory cells i.e. in the absence of appropriate stimulation of the stromal cells hemopoietic growth factor production declines to levels that are insufficient to activate nearby hemopoietic progenitors and these then enter a G_0 state. Alternatively, it is possible that stromal cells may also release specific inhibitory substances in a fashion that is subject to regulation. Recent data from the Terry Fox Laboratory have indicated that TGF-β1 can override the stimulation of hemopoietic progenitor cells that occurs in long-term marrow cultures following the addition of IL-1. Whether TGF-β1 is made by stromal cells and its possible involvement in the regulation of hemopoiesis by marrow cells will be very interesting to establish.

In summary, my studies have identified collagen-producing cells of the adherent layer as likely to be an important population for the maintenance and turnover of primitive hemopoietic cells. In addition, phenotypic and functional characterization of human marrow mesenchymal lines have suggested that production of hemopoietic growth factors may well be involved in the regulation of primitive hemopoietic cell proliferation. However, other molecules may also be involved. For example, further work is needed to

establish whether specific cell surface determinants exist that allow primitive cells to "home" to specific tissues. Similarly it is not known whether molecules that facilitate inter-cell interactions and thereby contribute to cell-mediated growth factor responses exist.

The availability of several cloned human marrow mesenchymal lines that exhibit the phenotype and regulated ability of cultured marrow cells to produce hemopoietic growth factors and synergistic activities should facilitate further studies of this type. Of particular importance will be future tests of the ability of these lines alone and/or in combination to reconstitute the hemopoietic supportive function of intact long-term marrow culture adherent layers.

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