D. Metcalf

Hemopoietic Colonies

In Vitro Cloning of Normal and Leukemic Cells

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With 54 Figures and 28 Tables

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Preface

The cloning of hemopoietic cells in semisolid medium began some 12 years ago, and when colonies of T= and B=lymphocytes were grown by several groups last year, the last major subclasses of hemopoietic cells had been successfully cultured in vitro.

The time seemed appropriate to write a short general account of this exciting new area of hematology, emphasizing particularly the potential value of these new techniques for direct studies on patients with leukemia and allied diseases.

I had hoped to refer to, or at least record, every paper published in this field, but the literature proved to be larger than I had realized, and furthermore, some areas were so active that chapters had to be rewritten over a period of less than a year.

I regret that what follows is an incomplete account and no doubt, by the time this book becomes available, the material will be even more out of date. However, I have described the basic techniques in some detail and have tried, where possible, to explain how and why the techniques can fail.

It is already possible to see patterns, perhaps even principles, emerging from this work which may well be applicable to other cell populations. These have been emphasized in the text in an attempt to make this review as creative as possible.

Like many biological techniques, semisolid cultures often fail at the worst possible times, and on such occasions life can be very frustrating. However, my colony colleagues around the world are a hardy, persistent lot not easily put down by adversity. The information they have amassed in the last decade is summarized in the pages to follow and, I think, reflects credit on their enterprise and industry.

Melbourne, November 1976

DONALD METCALF

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Chapter 1

Historical Introduction

Why attempt to clone hemopoietic cells in semisolid medium in vitro? As a general explanation, it can be said that the various hemopoietic populations are so inextricably intermixed and their regulatory systems so complex that analysis of hemopoiesis cannot be performed on intact animals. Furthermore, for the analysis of these populations in humans, the ability to manipulate the populations in vivo is essentially nonexistent for practical and ethical reasons.

While these considerations make it clear that hemopoietic populations must ultimately be analysed in vitro, why not use conventional liquid culture systems? Liquid culture systems certainly have many uses in hematology, but such systems are essentially "black boxes"—cell input and output can be monitored, but intermediate events are difficult and often impossible to follow. Furthermore, events cannot be related to individual cells.

The ability to grow clones of hemopoietic cells in semisolid medium permits an analysis of the proliferative and functional activity of individual cells and their progeny. How many progeny can one cell generate and are all cells similar in their capacity to proliferate and differentiate? Do they respond in an identical manner to specific regulatory factors? It will be seen in the account to follow that heterogeneity is in fact the characteristic of even apparently homogeneous hemopoietic cell populations and to recognize and measure this, it is essential that cellular proliferation can be followed on a clonal basis.

As will be discussed in the following chapters, one of the early disappointments in this field was the fact that colonies generated by leukemic cells are usually not distinguishable by shape or appearance from normal colonies. However, this is not universally true, and in some situations normal and leukemic cells produce distinctive colonies readily distinguishable in the same culture dish. In measuring the proliferation of normal versus leukemic cells in such a mixed population, the advantages of a cloning system become obvious.

The techniques for cloning hemopoietic cells in semisolid medium evolved from observations of tumor virologists on the growth in agar of normal and viral-transformed fibroblasts. In this work it was shown that transformed fibroblasts, unlike normal cells, were able to proliferate in the semisolid medium and form colonies of cells (see review by MACPHERSON, 1). Since the colonies were derived from single cells, i.e., the colonies were clones, this phenomenon offered a number of exciting possibilities to the experimental hematologist. With the use of semisolid medium it might be possible to work with cloned populations of leukemic cells and at the same time have a useful method for discriminating leukemic from normal cells. Indeed, it was shown that cells from tissue culture-adapted human lymphocyte cell lines (now known to be transformed by the Epstein-Barr virus) could be cultured in agar medium and could generate colonies of cells which proved of value in analyzing the clonal production of immunoglobulins [2].

Attempts to obtain clonal growth in agar of mouse leukemic cells were made simultaneously and independently by PLUZNIK and SACHS in Rehovot, using Rauscher virus-induced erythroleukemic cells, and by BRADLEY and METCALF in Melbourne, using AKR lymphoid leukemic cells. In both cases these initial attempts failed to obtain colony formation by leukemic cells. In an attempt to achieve success with the growth of the lymphoid leukemic

cells, the concepts of PUCK and his co-workers [3] were applied and the value investigated of using feeder layers of various cells, including spleen and bone marrow cells, in the culture system. Colony formation was obtained, but analysis of the system eventually showed that the colonies developing in the agar cultures were composed of neutrophilic granulocytes and/or macrophages and that the colonies were in fact derived from the normal hemopoietic cells included as feeder layers in the cultures [4]. In the cultures of erythroleukemic spleens, those colonies which did develop were similarly identified as normal neutrophilic and macrophage colonies [5] and the role of the spleen cells in supplying a necessary growth factor recognized.

Because the macrophages in these colonies actively phagocytose the metachromatic agar in the surrounding medium, these cells usually contain large amounts of metachromatic granular material. At the time of these early experiments, surface markers were not available for identifying hemopoietic subpopulations, and both groups experienced difficulty in excluding the possibility that the colonies of mononuclear cells were in fact mast cells. Indeed, the initial descriptions of these colonies by the Rehovot group referred to them as colonies of "mast" cells or "mast cell-like" cells [6, 7]. Because of these initial problems, subsequent workers in this field have been reluctant to identify metachromatic cells in colonies as mast cells, and despite the wide variety of culture systems subsequently developed, no descriptions of mast cell colonies have yet been made. It remains uncertain whether any of these techniques have succeeded so far in growing genuine mast cell colonies. However, as will be seen later, a typical culture of normal marrow contains a fascinating variety of colonies, and it is quite possible that mast-cell colonies are present in some of these cultures but have yet to be identified.

With the demonstration that clones of at least two hemopoietic subpopulations could be grown in semisolid agar from normal hemopoietic tissues, many workers became alerted to the possibility that other types of hemopoietic cells might similarly be able to be cloned in agar or comparable semisolid medium. However, from an analysis of the granulocyte-macrophage colony system, it became clear that cell proliferation was dependent on inclusion in the culture medium of a specific growth-stimulating factor (GM-colony-stimulating factor, GM-CSF) to stimulate the proliferation of this pair of related cell populations. Extrapolating from this information, it seemed likely that to obtain successful colony growth of other hemopoietic populations it would be necessary to include in the medium the corresponding regulators for the other cell types.

In an elegant series of experiments, AXELRAD and his colleagues [8, 9] modified the agar culture system, and using the specific erythropoietic regulator erythropoietin, demonstrated that erythropoietic colony formation could be obtained in semisolid plasma gel cultures. Subsequent work with this system has succeeded in growing erythroleukemic cells and indeed in obtaining rapid viral transformation in vitro of cloned erythropoietic cells [10, 11].

As no corresponding regulators were known for other hemopoietic cells, progress in cloning these other types of hemopoietic cells was dependent on chance observations. The agar culture technique for granulocytic and macrophage colonies was adapted by PIKE and ROBINSON [12] to permit the formation of similar colonies by human cells. In this system, underlayers of peripheral blood cells were used to supply the specific regulator, GM-CSF, to the target overlayer cells. Other workers modified the technique further by substituting methylcellulose for agar. In this latter system, it was noted by CHERVENICK and BOGGS [13] that some of the colonies developing were composed not of neutrophilic granulocytes or macrophages but of eosinophils. However, the complexity of peripheral blood underlayers with their mixed populations of cells has so far not permitted further analysis of this particular system.

Based on the principles emerging from the analysis of colony formation by normal mouse granulopoietic and macrophage-forming cells, systems were developed for cloning mouse myeloid leukemic and myelomonocytic leukemic cells in vitro [14, 15, 16]. In work with the latter system, it was noted that these myelomonocytic leukemic cells had the unique ability to stimulate normal marrow cells to form unusual colonies composed of loosely dispersed cells. The significance of this type of colony was not appreciated at the time.

Most of the evidence then available suggested that monocytes and macrophages were an important, perhaps the only, source of GM-CSF. However, studies by McNEILL [17] and PARKER and METCALF [18] showed that mitogen-stimulated lymphocytes were also a very rich source of GM-CSF. In the studies of PARKER and METCALF, it was noted that if the lymphocytes had been stimulated by pokeweed mitogen, the conditioned media also had the capacity to stimulate the same loose colonies as were noted previously in cultures stimulated by mouse myelomonocytic leukemic cells. The cells in these loose colonies were eventually identified as eosinophils, and it was shown that the active factor required for their stimulation (EO-CSF) was antigenically different and chemically separable from the GM-CSF stimulating the formation of colonies of neutrophils and macrophages [19]. More potent lymphocyte-conditioned media were subsequently obtained by the addition of 2-mercaptoethanol to the cultures. It was shown by METCALF and co-workers that highly active conditioned media of this type also were able to stimulate the formation of small colonies of very large cells which were identified as megakaryocytes [20].

During this developmental period, virtually everyone working in the field at some stage had attempted to grow colonies of normal lymphocytes in semisolid medium. These attempts were uniformly unsuccessful, and it was obvious that lymphocytes rapidly died after initiation of the cultures. Although achievement of the clonal growth of lymphocytes remained a "Holy Grail" of experimental hematologists, the problem was shelved temporarily as being too frustrating.

In work on the culture of granulocytic and macrophage colonies using unfractionated mouse peripheral blood, it was accidentally observed that if pleural cavity cells were mixed with the whole blood, colonies of normal fibroblasts developed in the culture medium [21]. Similar colonies could be grown from other hemopoietic populations. Parallel work in liquid cultures resulted in the demonstration that marrow and spleen stromal cells can form similar fibroblast-like colonies [22], and as shall be discussed later, these currently hold great promise as possibly being clones of cells producing specific hemopoietic regulators.

The agar culture system using whole blood supplements was used to study the fibroblast stromal cells in various mouse tumours with an unanticipated result. Some of the transplanted tumours used were mineral oil-induced plasmacytomas, and in cultures enriched by whole blood, it became obvious that plasmacytoma cells were also able to form colonies [23]. Use of supplemental whole blood or red cells in fact permits the clonal growth in agar of most mouse plasmacytoma cells—a cell type which had previously resisted culture attempts in semisolid medium unless first adapted to liquid cultures. At this time, it had recently been shown that addition of 2-mercaptoethanol to liquid cultures of lymphocytes and antibody-forming cells enhanced the proliferation of these cells [24, 25]. Tests on the effects of 2-mercaptoethanol in agar cultures of plasmacytoma cells similarly showed that colony formation by these neoplastic B-lymphocytes was often strikingly enhanced [26].

In retrospect, it is obvious that 2-mercaptoethanol should have been tested immediately in conventional agar cultures of normal mouse lymphoid cells, but the known inability of normal lymphocytes to survive in agar was a mental stumbling block which made such attempts apparently pointless. Eventually 2-mercaptoethanol was used by METCALF and co-workers in cultures of normal mouse lymphoid cells for other reasons and proved

spectacularly successful in permitting the clonal growth of normal B-lymphocyte colonies [27, 28] T-lymphocytes initially proved resistant to culture in single-step agar cultures, but as shall be described later, preliminary culture of lymphocytes in liquid culture with various T-lymphocyte mitogens permits colonies of T-lymphocytes to proliferate in agar [29]. Subsequent improvements in the culture medium now permit the single-step culture of T-lymphocytes (CLAESSON, M., unpublished data).

From this brief historical review, it will be apparent that cloning techniques are now available for all major hemopoietic subpopulations with the exception of hemopoietic stem cells. Efforts are now being made with the aid of these powerful tools to unravel the complexities of the interrelationships between hemopoietic subpopulations and the mechanisms controlling proliferation and differentiation both in normal and neoplastic hemopoietic cells.

At present, the technology for the culture of hemopoietic cells appears to be far in advance of the capacity to grow or clone *functionally active* cells from other tissues. If progress is to be made in understanding the proliferation and differentiation of cells from other tissues, these cells will eventually have to be grown and analyzed in comparable cloning systems. For biologists, it is instructive to consider whether any general principles have emerged from the development of cloning methods for hemopoietic cells which might be used to accelerate the development of comparable technology for other tissue cells. This question will be returned to in Chapter 14, but here it is worth making one further comment. The whole of the period for developing in vitro cloning techniques for hemopoietic cells occupied only 10 years, but progress was erratic, could rarely be planned, and most advances in fact were made accidentally. Nevertheless, the time intervals between the successful culture of each subpopulation progressively shortened (1965, granulocytes and macrophages; 1971, erythropoietic cells; 1972–1974, eosinophils; 1975, megakaryocytes; 1975, B-lymphocytes, T-lymphocytes). Faced with an almost complete lack of information on how to make high efficiency, primary cultures of, for example, functionally active pancreas or kidney cells, this accelerated rate of discovery of hemopoietic cloning methods is a hopeful sign.

The hardware of tissue culture—powdered media, disposable plastic culture vessels, filtration systems, sterilizers, incubators, and laminar flow work stations—have improved greatly in recent years, and it is now possible for most cell biologists to contemplate tissue culture approaches to their problems. It will become clear from the discussion to follow that many principles have emerged from the cloning work on normal and neoplastic hemopoietic cells which probably are generally applicable to other cell systems. The application of these principles can be expected to accelerate progress in understanding these other cell systems.

Analysis has shown that the various hemopoietic cloning systems have unusually high plating efficiencies and that quantitation of hemopoietic cells is both quick and relatively easy using these methods. What has emerged as an unexpected bonus from this work is the fact that these semisolid culture systems are particularly suitable for detecting and analyzing specific regulatory factors. The consequences of these properties of the cloning systems are that the analysis of normal and abnormal hemopoiesis has been revolutionized by the introduction of cloning methods.

It is the purpose of this monograph to describe the various semisolid culture techniques, to review the information produced by those techniques concerning normal and leukemic hemopoiesis, and to assess what problems might be soluble by further applications of in vitro cloning methods.

Chapter 2

An Outline of Hemopoiesis and Current Terminology

It is not within the scope of this monograph to deal with all aspects of hemopoiesis and the reader is referred to the book by METCALF and MOORE [30] for a more extensive discussion of the origin, development, and interrelationships between hemopoietic populations. However, to understand the implications of the results obtained with the in vitro cloning systems, it is necessary at this point to make a brief description of hemopoietic populations and introduce a number of terms to be used in the following chapters. In the interests of simplicity, the description to follow will not be annotated either by detailed evidence or source references.

A. Hemopoiesis

Because mature blood cells (red cells, polymorphs, monocytes, macrophages, eosinophils, platelets, and lymphocytes) have finite life spans, maintenance of stable levels of these cells in the circulation and tissues requires continuous cell production throughout adult life. The necessary cellular proliferation is broadly similar to that which was necessary in embryonic development to form the fully cellular hemopoietic organs from primitive anlagen.

The specific cell populations which proliferate and generate the mature blood cells are best described as consisting of three pyramidal-shaped compartments, or hierarchies, of cells (Fig. 1). The most ancestral cells are multipotential hemopoietic stem cells (stem cell compartment) which are capable not only of extensive self-replication (to form the stem cell compartment itself) but also of generating the various progenitor cells (progenitor cell compartment). Progenitor cells are possibly capable of a limited degree of self-replication but differ from stem cells in that their genomes are derepressed into one or other specific pathway of hemopoiesis, e.g., erythropoiesis or neutrophil-monocyte formation. Proliferation of progenitor cells leads to the formation of cells which for the first time are morphologically identifiable as belonging to specific hemopoietic populations, e.g., proerythroblasts, myeloblasts, etc. (morphologically identifiable compartment). Proliferation of these cells leads to the production of an expanded population of mature cells which appear in the peripheral blood and most of which not only are incapable of further proliferation but have relatively short life spans.

These various hemopoietic populations are located in the same organs except for the more mature lymphoid populations. Thus, in the normal adult, these populations are mainly located in the bone marrow with smaller numbers in the spleen and occasional cells of the first and second compartments being present also in the peripheral blood. In the developing embryo, hemopoietic populations appear first in the yolk sac and after migration into the embryo, are localized first in the liver, then subsequently populate the developing spleen and bone marrow.

The lymphoid populations require additional comment. Although lymphocytes arise from hemopoietic stem cells as do all other hemopoietic cells, two distinct populations of lymphoid

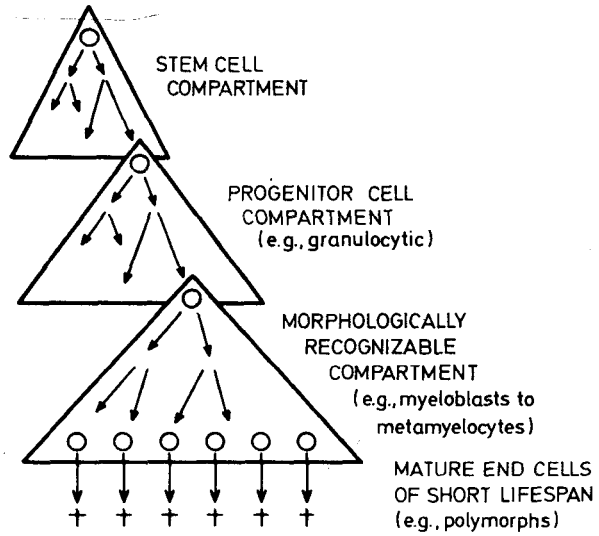

Fig. 1. Schematic representation of the three-tiered structure of hemopoietic populations

cells emerge—one generated mainly in the bone marrow (B-lymphocytes) and the other in the thymus (T-lymphocytes). Cells from the lymphoid progenitor cell compartment onward are located in these organs and also in the specialized lymphoid organs—the lymph nodes, Peyer's patches, tonsils, appendix, and spleen lymphoid follicles.

The important features of this three-tiered structure of hemopoiesis are: (1) the extensive capacity for proliferation and amplification within each compartment, (2) the progressive specialization from one compartment to the next, (3) the inability of cells to move from a mature compartment to a less mature compartment, e.g., of progenitor cells to revert to stem cells, (4) the inability of cells to move from one compartment to a compartment of corresponding maturation in another lineage, e.g., from the granulocyte progenitor cell compartment to the erythropoietic progenitor cell compartment, and (5) the progressive restriction in the final number of progeny able to be generated by cells in succeeding compartments.

As might be anticipated from this description, stem and progenitor cells are relatively infrequent in hemopoietic populations. For example, one cell in 1000 mouse marrow cells is a multipotential stem cell and one cell in 3–400 a granulocytic progenitor cell. Furthermore, members of the various hemopoietic compartments are mixed together in hemopoietic populations. In fact, hemopoietic populations exhibit no stratified architectural pattern (unlike organs such as the skin or testis) which would allow the most ancestral cells to be deduced and identified from their location in the tissue. Finally, stem and progenitor cells are relatively inconspicuous mononuclear cells with no morphological features allowing their certain identification. It follows from these considerations that selective assays are required to detect and enumerate the early members of these various hemopoietic subpopulations.

Hemopoietic stem cells can be identified in the mouse by their ability to generate colonies of cells in the spleen of syngeneic irradiated recipients [31], a property responsible for the other name for these cells—spleen colony-forming cells or units (CFU or CFU-S). No corresponding assays for stem cells exist in other animals or man although their proliferative

potential in these species can be deduced indirectly from their capacity to repopulate the hemopoietic tissues of irradiated recipients or recipients depleted of hemopoietic populations for other reasons.

Progenitor cells can be detected and enumerated by their capacity to form colonies in the various in vitro cloning systems to be discussed shortly. These studies have given rise to a somewhat confusing variety of names and abbreviations for progenitor cells, e.g., colony-forming cells (CFC), CFC agar (CFA-A), CFC culture, CFU-C etc.

Morphologically recognizable cells and their progeny can be identified by classical morphological methods which can now be supplemented by sequential studies on pulse-labeled populations. The special names for the various cells within each hemopoietic family (e.g., myeloblasts, myelocytes, metamyelocytes, polymorphs) can be obtained from any standard hematological textbook.

The yolk sac is the only location in which hemopoietic stem cells are generated de novo. Following migration of these cells into the embryo, the supply of stem cells throughout life is maintained by the capacity of these initial cells for extensive self-replication (30). So extensive is this capacity that there are no situations in which stem cell supplies appear to fail, and there are no proven examples in late embryonic or adult life in which new hemopoietic stem cells are generated from other cells. Most stem cells remain quiescent in a non-cycling state throughout life, and there is evidence to suggest that hemopoiesis is maintained by the sequential activation, expansion, and eventual exhaustion of limited numbers of these stem cells.

Within the morphologically-recognizable compartment there is a sequence through which cells pass, e.g., myeloblasts to myelocyte to metamyelocyte to polymorphs, each population being larger in size than the preceding. However, within the stem and progenitor cell compartment, the exact situation is unknown. Cells entering these compartments and proliferating can be assumed to generate increasing numbers of progeny, but the manner in which they leave the compartment is not known. Conceivably, any cell within one of these compartments might be capable on a purely random basis of undergoing selective genetic derepression or activation and entering a succeeding compartment. Alternatively, as is the case in the morphologically recognizable compartment, a specific sequence of events may be associated with or determine exit from the compartment, e.g., the number of preceding divisions the cell has undergone.

Two facts have been established about cells in the stem and progenitor cell compartments: (1) commitment of multipotential stem cells to a specific pathway of differentiation, e.g., the stem cell to progenitor cell sequence is under control of what appear to be "microenvironmental" factors, and (2) proliferation of progenitor cells and their progeny in the succeeding morphologically recognizable compartment is mainly under control of specific humoral factors, e.g., erythropoietin, GM-CSF, etc.

The existence of local or microenvironmental regulatory factors can be deduced from the restricted localization of hemopoietic tissues in the body; from the segregation of hemopoietic populations in the spleen, and from the initial relatively pure populations of hemopoietic cells in spleen colonies generated by CFUs in the spleen. While most agree that commitment of stem cells to one or other type of progenitor cells is a stochastic event, opinions differ on the likely mechanism. Some favor the concept of fixed cellular niches in the hemopoietic tissue with stromal cells or microenvironmental cells forming such niches and functionally differing from cells forming adjacent niches. Random entry of stem cells into an erythropoietic niche would result not only in continuing self-generation on the part of the stem cell but in the transformation of some of the progeny to erythropoietic progenitor cells. The process envisaged might involve cell contact between stem cell and specific

receptor sites on the microenvironmental cells. Others envisage that these microenvironmental or "managerial" cells might not be arranged as fixed structures but might be dispersed throughout the tissue and generate short range gradients of specific factors which could trigger entry into an appropriate pathway of differentiation following binding by the stem cells of a significant concentration of the specific factor.

Either process would account for the observed data, the only point of practical importance is that so far it has not been possible to recreate these events in simplified in vitro systems. As a consequence, little is known about the nature of the stem cell to progenitor cell sequence.

With the introduction of in vitro cloning techniques, similar events from the progenitor cell to end cell stage can now be analyzed in isolation in vitro. It is quite apparent that proliferation within the progenitor and morphologically recognizable cell compartments is dependent on adequate concentrations of the appropriate specific regulatory factor. While this makes in vitro analysis relatively simple, it must be cautioned that in vivo events may not necessarily be as simple. It may well be, for example, that microenvironmental factors can also operate on the more mature compartments (just as humoral factors might well influence the stem cell to progenitor cell transition). This reservation should be kept in mind in reading the subsequent chapters. As will be seen, events are complex enough even in vitro but it is possible that even the in vitro systems do not genuinely reflect the full complexity of events occurring in vivo.

B. Terminology

To assist the reader in the following chapters a master list has been compiled in Table 1 of the names and abbreviations of the various hemopoietic cells and regulators. The terms used throughout this book are a combination of those in most common usage and a new systematic terminology for progenitor cells and their regulators. Names used by other workers are included in the synonym list.

C. Glossary

Certain names and terms will be found repeatedly in the text to follow. For the novice, an explanation of some of these terms is now given:

B-Lymphocytes. Subpopulations of lymphocytes mainly involved in humoral immune responses (mediated by immunoglobulin production). Although all synthesize immunoglobulin, plasma cells are a more specialized subset in which immunoglobulin production is extremely active. Derived from progenitor cells in bone marrow (also from the bursa of Fabricius in chickens), but the name is applied regardless of where cells are subsequently located. Note that both T- and B-lymphocytes proliferate extensively in lymph nodes, spleen, and Peyer's patches (that is, in the so-called secondary lymphoid organs). B-lymphocytes are identifiable by membrane immunoglobulin, antibody production, membrane receptors for antigen, the Fc portion of immunoglobulin, and the C3 component of complement.

Table 1 Names and abbreviations of hemopoietic cells and regulators

Cells			Regulatory factor		
Name	Abbreviation	Synonyms	Name	Abbreviation	Synonyms
Stem cell compartment					
Hemopoietic stem cells	HSC	Spleen colony-forming units (CFU-S or CFU)	Microenvironmental regulatory factors		Hemopoietic inductive microenvironment (HIM)
Progenitor cell compartment (committed stem cell compartment)					
Neutrophil and macrophage progenitor cells	GM-CFC	Granulocyte-macrophage colony-forming cells (GM-CFC); in vitro colony-forming (in vitro CFC); colony-forming units, culture (CFU-C); colony-forming units, agar (CFU-A)	Granulocyte-macrophage colony-stimulating factor	GM-CSF	Colony-stimulating activity (CSA), macrophage-granulocyte inducer (MGI)
Macrophage progenitor cells (peritoneal type)	M-CFC	Macrophage colony-forming cells	Granulocyte-macrophage colony-stimulating factor	GM-CSF	
Eosinophil progenitor cells	EO-CFC	Eosinophil colony-forming cells	Eosinophil colony-stimulating factor	EO-CSF	
Megakaryocyte progenitor cells	MEG-CFC	Megakaryocyte colony-forming cells	Megakaryocyte colony-stimulating factor	MEG-CSF	?Thrombopoietin
Erythropoietic progenitor cells	E-CFC	Burst forming units erythroid (BFU-E)	Erythropoietic colony-stimulating factor	E-CSF	Erythropoietin (Epo)
B-lymphocyte progenitor cells	BL-CFC	B-lymphocyte colony-forming cells	?	?	?
T-lymphocyte progenitor cells	TL-CFC	T-lymphocyte colony-forming cells	?	?	?

Chalone. By the original definition, a product of mature cells specifically inhibiting cell proliferation in that cell lineage. Often used loosely for an inhibitor of uncertain origin with usually incompletely characterized inhibitory activity on a particular cell system.

Clone. A cell population derived from the proliferation of one ancestral cell.

Cloning. The process of initiating a population in culture from a single cell. Strictly speaking, the cell should be micromanipulated to a fresh culture or isolated by a ring from other cells. Since most colonies in semisolid medium are clones, the term is often used loosely to describe discrete colony formation of only one cell type by cells contained in a mixed population of cells.

Clusters. Aggregates of cells formed by proliferation in vitro usually containing 3–50 cells.

Colonies. Aggregates of cells formed by proliferation in vitro and usually containing more than 50 cells. For human colonies, a lower limit of 40 cells is commonly used. Some workers use a limit of 20 cells. The criteria used for colonies are important for the interpretation of the data and should be noted with care in reading papers in this field.

Commitment. Usually used in reference to the stem cell to progenitor cell transition. Denotes loss of multipotential state, acquisition of synthetic activities for one special subpopulation and presumably the acquisition of membrane receptors for the specific regulator. Most likely mechanism, selective derepression of the genome.

Colony Stimulating Activity (CSA). A curious term used by some workers to denote the specific chemical factor stimulating neutrophilic and macrophage proliferation in vitro. Use best restricted to impure material with one known biological activity but probably containing a mixture of specific factors.

Conditioned Medium. Medium harvested from cultures of pure or mixed cells and containing many additional substances secreted by, or released from, these cells. Sometimes referred to as "spent" medium although such medium usually still contains adequate levels of nutrients. The growth inhibition sometimes observed with these media is often due to additional toxic or inhibitory factors derived from the cells.

Differentiation. Somewhat broader term than commitment, conveying concept of both commitment and maturation.

End Cells. Cells having reached full cytoplasmic maturation and incapable of further division. Death usually follows shortly after. Note that with lymphoid cells, end cells can avoid subsequent death if restimulated to proliferate by the appropriate antigen. Macrophages may also enjoy this alternative to a limited degree.

Factor. In the present context usually used to refer to a specific chemical substance with a definable property, e.g., the ability to stimulate the proliferation of certain cells, e.g., colony-stimulating factor. The term is an operational one only until chemical extraction procedures have succeeded in purifying the molecule concerned.

Families or Subpopulations. Population subcompartments of one morphological type, e.g., erythropoietic.

Granulocytes. Usually used to denote neutrophilic granulocytes (neutrophils or polymorphs) and less often to denote their immediate morphologically recognizable ancestors. Although eosinophils and mast cells also have granules these cells are usually not covered by the word granulocyte.

Hemopoiesis. Process of formation of blood cells. In this book (and usually) also meant to include the formation of lymphocytes since these are blood cells.

Humoral Factor. A humoral factor is literally something blood-borne, i.e., something detected in the blood, but of course the same factor might also be present in, or produced by, various tissues. The virtue of the expression is to denote that it is a chemical substance which leaves the cell and can act at a distance, traveling to the target cells via the blood. All hormones are humoral factors. Humoral factors may possibly differ from hormones in originating from more than one tissue or cell type. The term is often used loosely to refer to products releasable from cells and theoretically able to act at a distance, e.g., as in conditioned medium but strictly this remains hypothetical until proof is obtained of the presence of the factor in the peripheral blood.

Lymphopoiesis. The specific formation of lymphocytes and usually used to refer to events from the progenitor cell stage onward when cells already have some or all properties of lymphocytes or plasma cells.

Maturation. The progressive sequence of cellular events leading to accentuation of or specialization in one particular activity, e.g., hemoglobin formation or phagocytic activity. Often associated with loss of the capacity for further cell division and used loosely to refer to this state.

Monocyte-Macrophages. Collective term used to cover both monocytes and macrophages. Most tissue macrophages are derived from nonocytes produced in the marrow and spleen which seed in the tissue via the circulation and transform there to macrophages without further proliferation.

Myeloid. Derived from marrow. Often used as synonymous with neutrophilic granulocytes, e.g., as in myeloid leukemia.

Plating Efficiency. Strictly speaking, the percentage of a uniform population of cells able to proliferate in vitro. Often used incorrectly to describe the frequency of proliferating cells in cultures of a mixed cell population.

Poiesis. Formation, e.g., erythropoiesis—formation of red cells.

Poietic. Capable of forming, e.g., erythropoietic—capable of forming erythroid cells.

T-Lymphocytes. Subpopulations of lymphocytes mainly involved in cell-mediated immune responses, responses to viruses and in collaborative interactions with B-lymphocytes. Derived from progenitor cells located in the thymus, but the term applies to these cells wherever they are subsequently located in the body. Identifiable by surface theta antigens, rosette formation with red cells etc.

Underlayers. (Feeder layers) layers of cells incorporated under the cells being cultured, often in thicker agar, to supply growth factors to the cultured cells.

Chapter 3

Techniques for the Clonal Culture of Hemopoietic Cells in Semisolid Medium

Many different methods have now been developed for obtaining colony formation in semisolid agar medium by animal or human hemopoietic cells. Rather than provide an extensive description of all current methods for each separate type of hemopoietic cell, it is proposed to give a general discussion of the principles and to describe one standard method for each cell type. References to other methods will be given where these alternative methods have advantages for special purposes.

A. General Principles

The cloning techniques to be described are designed to obtain *primary* cultures of hemopoietic cells taken directly from animals or patients. When used effectively, they provide information on the number and functional activity of hemopoietic cells in vivo at the time the samples were removed for culture. These solid state cloning systems are not particularly useful for subculture studies, and the long-term behaviour of hemopoietic cells in this type of culture has not been extensively investigated.

The culture systems differ from conventional liquid culture systems in being single-step processes. The cells are suspended in a semisolid culture medium (usually in Petri dishes), and the cultures are incubated without subsequent media changes or supplementation until scoring of cultures is performed—usually 7–14 days later. Since refeeding of cultures or serial transfer of cultured cells are usually not involved, the requirements for performing this type of tissue culture are not as stringent as for other types of culture. As antibiotics are used in the culture medium and replicate cultures invariably performed, loss of occasional cultures from contamination is not critical. Because of this, it is possible to perform this type of culture in a reasonably clean room without special tissue culture facilities, e.g., filtered air or UV lights. Of course, if laminar flow hoods or tissue culture rooms are available, so much the better, but lack of these facilities certainly does not prevent such culture work.

The general requirements for tissue culture-washed glassware, apparatus for filtration of media, and small samples of test material are the same as for other types of tissue culture.

B. Collection of Cells

Marrow cells from mice or other laboratory animals are collected after killing the animal by cervical dislocation or with ether. The skin is cleaned with 70% alcohol, and using boiled instruments, the muscles are carefully stripped from the femur. Holding the tibia at right angles to the femur and pulling along the axis of the femur, the knee joint and lower end of