Controlling the Production of Blood Cells

By Martin J. Cline and David W. Golde

The development since 1966 of a technology for growing stem cells in vitro has provided new insights into the controls of blood cell production. Hematopoietic hormones have been purified and important cellular interactions in hematopoiesis have been defined.

The rapid accumulation of information in the field of hematopoiesis has increasingly required reevaluation of the traditional concepts of the controls of blood cell production. This expansion has been so rapid that in the past year alone conferences devoted to synthesizing and analyzing this body of information have taken place at Cold Spring Harbor, Keystone (Colorado), Brookhaven, and Seattle. Although these conferences addressed many issues, certain questions were an intrinsic element of all: (1) What are the controls that keep the circulating blood cells at a “normal” level? (2) What are the mechanisms for altering the supply of blood cells in response to widely fluctuating demands? (3) How does the bone marrow meet competing demands for erythropoiesis, granulopoiesis, and megakaryocyte production when all elements are reduced by chemotherapy or irradiation? (4) What regulates hematopoietic cell differentiation and gene expression? (5) What goes wrong with the normal control mechanisms in hematologic malignancy?

Although the answers to these questions are still not available in a form that is intellectually satisfying or useful in developing strategy for the treatment of disease, we are beginning to develop partial answers, model systems, and new technology that ultimately will provide information directly applicable to the therapy of human disease.

New Technology

The methods for isotopically labeling blood cells and determining various compartment sizes and turnover rates in intact animals and man were largely established by the mid-1960s. Although important studies were still to be done, the kinetic approach in vivo had limitations with regard to studying stem cell function. In 1966 Bradley and Metcalf in Australia and Pluznik and Sachs in Israel described a system for growing granulocyte-macrophage colonies from bone marrow suspended in soft agar. The culture system in vitro had three requirements: responsive stem cells from bone marrow or other hematopoietic tissue, a hematopoietic hormone to serve as stimulus, and semisolid matrix that would support growth and keep the progeny of a single cell together as a “colony.” The number of colonies formed was shown to depend on the number of progenitor cells and the concentration of the stimulus in the culture dish. Clearly, such a system could be
Table 1. Glossary of Terms Used in Current Hematopoietic Research

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>Clonogenic assay</td>
<td>An assay for clones or colonies arising from a single stem cell</td>
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<td>Stem cell</td>
<td>A primitive cell capable of self-renewal and differentiation</td>
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<td>Commitment</td>
<td>The decision of a stem cell to differentiate along a single pathway, e.g., erythroid</td>
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<tr>
<td>CFU-S</td>
<td>Colony-forming unit-spleen: a measure of the pluripotent stem cell for erythroid, granulocyte, megakaryocyte, and eosinophil development</td>
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<tr>
<td>CFU-C (CFU-G,M)</td>
<td>Colony-forming unit-culture (also called colony-forming unit-granulocyte/macrophage): forms colonies in agar</td>
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<tr>
<td>BFU-E</td>
<td>Burst-forming unit-erythroid: a primitive RBC precursor</td>
</tr>
<tr>
<td>CFU-E</td>
<td>Colony-forming unit-erythroid: immediate antecedent of the pronormoblast</td>
</tr>
<tr>
<td>CFU-eos, M, TL, etc.</td>
<td>Colony-forming unit-eosinophil, megakaryocyte, T lymphocyte, etc.</td>
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<tr>
<td>CSF</td>
<td>Colony-stimulating factors: a family of glycoproteins stimulating CFU-C (also called colony-stimulating activity (CSA))</td>
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used to explore the nature of the stem cells and of the controlling hormones. Over the next 12 years techniques were evolved for growing many classes of hematopoietic precursor cells—granulocyte and macrophage, erythroid, eosinophil, megakaryocyte, and B and T lymphocyte. This new technology gave rise to a new nomenclature, which we have summarized in Table 1.

It should be noted that these “clonogenic” techniques measure only committed stem cells, i.e., stem cells that can develop along only a single pathway, such as erythroid. Thus far, only limited success has been achieved in growing in vitro the pluripotent stem cells that give rise to several cell lines. In general, the clonogenic cultures are short term, keeping cells alive and proliferating for a few days or a few weeks. Another facet of the new technology, still in evolution, is the use of liquid culture techniques that will preserve stem cells for long periods of time. In the most common type of long-term culture system, adherent cells from mouse bone marrow are used to form a “stroma” on the surface of a plastic culture vessel. The stroma consists of epithelioid cells, macrophages, and giant fat-containing cells and probably other cell types as well. After several weeks in vitro the adherent cells are “recharged” with a fresh inoculum of bone marrow cells. These continue to generate pluripotent and committed stem cells. At the moment, the limits of this approach are measured in weeks to months.

Still a third approach in the new technology is the use of established cell lines to measure the controls of cell differentiation, response to hematopoietic hormone, or the production of hematopoietic stimulators and inhibitors. Perhaps the most important of these permanent cell lines in current use is the murine Friend erythroleukemic line. Disease in mice is caused by a complex of viruses and is manifested as leukemia associated with polycythemia or anemia. Certain clones of established Friend virus–infected cells growing in vitro undergo maturation along the erythrocyte pathway on exposure to a variety of relatively simple chemicals such as dimethylsulfoxide, hexamethylene bisacetamide, or butyric acid. With such exposure, a high percentage of cells transform from primitive “blast” cells, manifesting few differentiated characteristics, to cells that manufacture markers of differentiated red cells including globin messenger RNA, spectrin, glycoporphin, and ultimately hemoglobin. Until recently these Friend leukemia lines were unique in showing such inducible differentiation; however, Collins et al. recently described a human cell line from a patient with promyelocytic leukemia that also differentiates in vitro in response to simple chemical signals. Other investigators have
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described a continuously growing human cell line that responds to colony-stimulating factor (CSF). These lines provide model systems for studying the signals for differentiation and the mechanisms of action of hematopoietic hormones.

What are the key points that have been learned from the application of this new technology in the past few years?

Fig. 1. Scheme of stem cell differentiation. (A) Lymphoid and myeloid development from a common pluripotent stem cell. (B) Analogous to Fig. 1A but showing progenitor cells defined by clonal assay. CFU-D, granulocyte/monocyte progenitor detected in peritoneal diffusion chambers; see Table 1 for other abbreviations.
The Stem Cell Is Real

A hematopoietic stem cell is a primitive cell capable of producing copies of itself as well as daughter cells committed to maturation. For example, an erythroid stem cell can reproduce itself but also gives rise to pronormoblasts. Stem cells that can produce daughters of various cell lines are pluripotent; those that produce daughter cells of only a single line are unipotent. The overwhelming weight of evidence indicates that pluripotent stem cells do indeed exist. This evidence has been of three types: first, the appearance of marker chromosomes in the blood cells of irradiated mice given infusions of compatible marrow; second, the distribution of the Philadelphia chromosome in leukemic granulocytes, megakaryocytes, monocytes, and erythroid precursors; third, the distribution of marker isozymes of glucose-6-phosphate dehydrogenase (G6PD) in certain myeloproliferative disorders. The scheme pictured in Fig. 1A is a representation of our current knowledge of the interrelationships among stem cells; Fig. 1B shows the corresponding assays for measuring these stem cells. One should note that there is a close relationship between neutrophilic granulocyte and monocyte stem cells, closer, for example, than that between neutrophils and eosinophils. Good evidence from Phillips’ laboratory at the Ontario Cancer Institute indicates that myeloid stem cells (CFU-S) and lymphoid stem cells are indeed related to each other; Abramson et al. found some radiation-induced chromosome markers distributed in CFU-S, T cells, and B cells, but others were distributed only in CFU-S and still others only in T lymphocytes. The relationships outlined in Fig. 1A must still be considered tentative; they may have to be redrawn as new knowledge becomes available.

Several obvious questions come to mind: What do pluripotent stem cells look like? Are they usually dividing? If not, then what factors induce their proliferation? What tells a stem cell to make a commitment, i.e., what instructs the pluripotent stem cell to become an erythroid stem cell or a megakaryocyte stem cell? We have only partial answers to these key questions.

A pluripotent hematopoietic stem cell is detected by the mouse spleen colony assay (CFU-S). It is found in the light-density cell fraction of mouse marrow and spleen among the lymphocytes, and probably looks like a small lymphocyte. Under ordinary circumstances, only a small fraction of CFU-S are actively dividing. This fraction rapidly increases when there are demands to expand the pluripotent stem cell compartment, as after subtotal irradiation or bone marrow transplantation. There is limited information available regarding the regulators of pluripotent stem cell proliferation, but a recent model developed by Iscove postulates the existence of another hematopoietic hormone affecting the most primitive stem cells. We do not know what triggers commitment of pluripotent stem cells to a single line of development. Three different theories suggest that the primary determinants are (1) simply random events that dictate that with each cell division the stem cell has an equal opportunity of becoming a red cell precursor, a neutrophil precursor, and so forth, (2) local microenvironmental influences within the stroma of the bone marrow or the spleen that program the direction of stem cell development (e.g., red cells develop more readily in mouse spleen than in mouse bone marrow after subtotal irradiation and marrow transplantation), (3) hormones operating at long range may interact with receptors at the surface of stem cells to orient their commitment and subsequent development.
arguments favoring all three theories, and the mechanism of stem cell commitment to a given line of differentiation remains one of the primary questions in hematopoiesis today.

**Cellular Interactions**

Anyone looking at a bone marrow smear is struck by the heterogeneity of cell forms. The morphologic heterogeneity in a sense reflects the diverse functions performed by blood cells: oxygen and CO₂ transport, phagocytic defense function, antibody and lymphokine production, and hemostasis. Why are all these diverse cells packaged together in the bone marrow? The answer probably lies in the phylogeny of the development of blood elements in various species and at different times through the millenia. Our current concept is that critical cellular interactions govern the proliferation and differentiation of hematopoietic stem cells. The interactions that have recently been described are summarized in Table 2. Although most of these have been described in systems in vitro, some undoubtedly have relevance for the whole animal.

**Erythropoiesis**

For the past two decades, textbooks have indicated that the major control of red cell production resides in a glycoprotein hormone, erythropoietin, elaborated in the kidney under the control of an oxygen-sensitive cell and interacting with early red cell precursors in the bone marrow. Erythropoietin has probably been purified to homogeneity, and a radioimmunoassay may be close at hand. These developments will allow for an increase in our understanding of erythropoietic regulation. The systems in vitro have already provided considerable new information. For example, it was found that the most primitive identifiable erythroid stem cell is relatively insensitive to erythropoietin. This cell, designated the burst-forming unit–erythroid (BFU-E), makes large clusters of erythroid colonies but only in response to levels of erythropoietin that are some 50–100 times those usually encountered in the plasma. The BFU-E is not normally in cell cycle but appears to enter cycle only when induced by general stimuli to marrow regeneration such as irradiation. These observations suggest that most of the controls of red cell production are normally mediated by the interaction of erythropoietin with CFU-E, which are the progeny of BFU-E and which are the immediate precursors of the basophilic normoblast. A burst-promoting activity has been identified that may modulate the growth of the

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### Table 2. Recently Described Cellular Interactions in Hematopoiesis

| Interaction | Ref
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<tr>
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<tr>
<td>T lymphocytes influence BFU-E</td>
<td>28, 29</td>
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<tr>
<td>T lymphocytes influence CFU-S</td>
<td>30</td>
</tr>
<tr>
<td>Stimulated lymphocytes produce CSF</td>
<td>31, 32</td>
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<tr>
<td>Mononuclear phagocytes produce CSF</td>
<td>33, 34</td>
</tr>
<tr>
<td>T lymphocytes stimulate eosinophilopoiesis</td>
<td>35</td>
</tr>
<tr>
<td>Pokeweed mitogen-stimulated spleen cells stimulate colonies with mixed cell lines</td>
<td>10</td>
</tr>
<tr>
<td>Granulocytes and macrophages produce inhibitors of granulopoiesis</td>
<td>36</td>
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most primitive erythroid progenitors. Erythropoiesis is also stimulated by nonhe-
matopoietic hormones such as thyroid, steroids, and growth hormone.38,39

The current concepts of the controls of red cell production do not address certain
key question: Once a stem cell has committed to erythropoiesis, what controls the
synthesis of the messenger RNA that characterize the red cell precursor? For
example, what controls the hemoglobin A to hemoglobin C switching in sheep?
More importantly, what controls the switch from messenger RNA for fetal
hemoglobin to that for adult-type hemoglobin in postnatal human life? The answers
are unknown.

Granulopoiesis

The recent acquisition of knowledge about the controls of granulopoiesis has
been dramatic. The key recent advances are these: First, the primary candidates for
the homones stimulating granulocyte production in vivo are the colony-stimulating
factors (CSF) necessary for growth of granulocyte-macrophage colonies in vitro.
These appear to constitute a family of glycoproteins, each with relative specificity;
some stimulate mainly granulocyte colonies, others macrophage colonies, and still
others mixed colonies. These hormones appear to be antigenically related to each
other. Two mouse CSF preparations have been purified to homogeneity, and
human placental CSF has been highly purified but is not yet homogeneous.40,41 In
the mouse a variety of tissues serve as sources of CSF; for man, the main tissues are
macrophages, lymphocytes, and placenta. Recently a radioimmunoassay for CSF
was developed, and human cell lines are now available that produce CSF.

Just as there is a family of CSF, so too there exists heterogeneity among
colony-forming cells. These are closely related but distinctive cell subpopulations
that vary in size and density. The hormonal stimuliators of eosinophil production
appear to be distinct from those for neutrophils. Antigen- or mitogen-stimulated
lymphocytes appear to be the primary source of CSF eosinophils.

Thus far, all secure evidence points to positive hormonal control of granulocyte
production. Hypotheses concerning negative-feedback control of granulocytes are
attractive, and several products of mature granulocytes and macrophages, such as
prostaglandins of the E series,28 have been shown to inhibit granulopoiesis in vitro.
The relevance in vivo of these diverse products remains, however, to be
defined.43

Clinical Disease

The new technology of hematopoietic cell culture has been widely applied in the
clinic with the hopes of understanding disease and shedding light on normal
physiology. Unfortunately, the techniques have often been applied before their
complexity was been wholly appreciated, with the result that clinical research in
hematopoiesis in vitro has produced a hodge-podge of truths, half-truths, and
untruths. It therefore is imperative to ask at this juncture, what is the knowledge of
disease of which we are reasonably certain? What questions can we pose with
clarity? We believe that the following are the knowns and unknowns.

Polycythemia rubra vera. This is a clonal disorder in which the abnormality
arising in a single cell ultimately affects multiple hematopoietic cell lines.21 We do
not know why the pool of erythroid precursors is expanded in polycythemia vera. The old concept that polycythemia vera represents autonomous erythropoiesis uncontrolled by erythropoietin seems untenable. In this disease, at least some erythroid precursors retain responsiveness to erythropoietin in vivo and in vitro. The question of possible heightened sensitivity of polycythemic BFU-E or CFU-E to erythropoietin remains unanswered.

Preleukemia. The disease syndrome of preleukemia, in which multiple abnormalities of hematopoiesis antedate the development of overt acute myeloid leukemia, also appears on the basis of cytogenetics to be a clonal disease. Abnormalties of cell maturation in vitro also antedate development of overt leukemia and may aid in diagnosis. The evidence is suggestive, but not definitive, that normal hematopoietic clones are severely depressed or absent during the preleukemic phase and subsequently during chemotherapy. The signals for the evolution of preleukemia to frank leukemia are unknown.

Aplastic anemia and Diamond-Blackfan syndrome. The 1976–1977 model that aplastic anemia and Diamond-Blackfan syndrome are caused by autoggressive lymphocytes may not be tenable, and the evidence is not standing up to hard scrutiny. Most of the early results probably reflected technical artifacts or the results of alloantigen sensitization by transfusion. The best model of bone marrow failure remains that of Morley and Blake, who showed that the stem cell injury induced by busulfan is long sustained and may be too subtle to be picked up by the routine blood count.

Acute myeloid leukemia (AML). The observation that many leukemic populations may respond to CSF and even show some maturation in vitro has led to the repeated suggestions that leukemic cells are not all that bad, it’s just the environment that is wrong. More critical analysis, however, suggests that this is not the case. The fundamental defect in AML appears to be an intrinsic abnormality in the cell’s ability to undergo terminal differentiation, with the result that the blast cells accumulate and ultimately kill the host.

The concept that the pattern of growth in vitro of leukemic cells is clinically useful in predicting prognosis or guiding selection of chemotherapeutic agents has still to be established.

Future Directions

In hematopoietic research, as in many other fields of biologic investigation, advancement is by a series of steps rather than a smooth upward curve. The steps are carved by single advances in technology or, more rarely, by conceptual advances. The future evolution of our current “step” seems clear. A multiplicity of hematopoietic hormones will be purified and their biologic effects on purified stem cell populations will be defined. When available in sufficient quantity, these hormones will undergo clinical trial. A large effort will be directed toward perfecting long-term marrow culture systems. If successful, this will allow us to explore the still hazy world of close cellular interactions. Perhaps it will also allow us to grow stem cells in vitro for clinical use. Understanding leukemia will require understanding the normal controls of stem cell commitment and differentiation. The tools to carve this next step upward may be at hand, with model systems of established hematopoietic cell lines and with recombinant DNA research.
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