Clonogenic Cells in Acute Myeloblastic Leukemia

By James D. Griffin and Bob Löwenberg

NORMAL granulocytes, monocytes, and erythrocytes are derived from small numbers of bone marrow progenitor cells that have extensive proliferative capacity. In vitro cell culture techniques to identify progenitor cells have been available for 20 years and depend on the ability of these cells to form colonies of fully differentiated cells in semisolid medium in response to specific growth factors. These colony assays have been used to investigate in vitro regulation and various biological characteristics of both multipotent and lineage-committed progenitor cells (see reviews 7 to 9). Shortly after development of normal colony culture techniques these methods were applied to acute myeloblastic leukemias and culture conditions were defined that allowed growth of colonies of leukemic cells. In most cases, formation of leukemic colonies was not autonomous, and addition of growth factor(s) from normal leukocytes augmented colony growth. It was surprising, however, that despite the apparent homogeneity of most populations of acute myelogenous leukemia (AML) cells, only a small fraction of cells had the capacity to proliferate in vitro.

These clonogenic leukemic cells have been the subject of considerable investigation, and also the source of some controversy, in the ensuing years. The possibility that these clonogenic cells act as stem cells in vivo to maintain the rest of the leukemic cell population is an intriguing possibility that has potentially important therapeutic significance. The identification, culture, growth characteristics, and biological and clinical significance of clonogenic cells in AML will be the subject of this review.

NORMAL HEMATOPOIETIC STEM CELLS

Primitive pluripotent hematopoietic stem cells with the capacity to form all blood elements, including myeloid cells, erythrocytes, megakaryocytes, and lymphocytes, have been demonstrated in mice and humans using genetic markers. Murine granulocyte/erythrocyte/megakaryocyte multipotential progenitor cells can be readily assayed as spleen colony-forming units (CFU-S), and these cells have been shown to have at least limited self-renewal capacity in secondary colony assay experiments. In both mice and humans an in vitro colony assay for a progenitor cell common to granulocytes, erythrocytes, monocytes, and megakaryocytes has been developed (CFU-GEMM), and in some circumstances T lymphocytes are also generated. The factors regulating proliferation of this cell are poorly understood, but T cell–derived lymphokines are required in vitro. The CFU-GEMM is a rare cell in normal human bone marrow (approximately 5 to 10 cells per 10^6 mononuclear cells), and despite some similarity to the murine CFU-S, self-renewal capacity is minimal or absent. The progeny of CFU-GEMM cells become committed to one lineage or another, and these committed progenitor cells can be assayed in vitro as erythroid burst-forming units (BFU-E), erythroid colony-forming units (CFU-E), granulocyte/monocyte colony-forming units (CFU-GM), or megakaryocyte colony-forming units (CFU-Mega). The humoral factors inducing colony formation in each case are partially defined. The CFU-GM cell, for example, proliferates in response to colony-stimulating factors (CSFs), a family of glycoproteins secreted by activated T cells, monocytes, endothelial cells, and certain tumor cells. In general, proliferation of any of the committed progenitor cells is associated with differentiation, although colonies of undifferentiated human blast cells have been described. The distinction between various hematopoietic growth factors has not been adequately made in all cases, however, and definition of the range of activity of each factor may require studies with factors produced by molecularly cloned genes. For example, recent studies with recombiant human GM-CSF have shown that this CSF stimulates growth of not only CFU-GM, but also BFU-E and CFU-GEMM. It is also likely that direct cell-cell interactions are important in the regulation of hematopoiesis, and stromal cells, endothelial cells, natural killer cells, and T cells may all play roles. However, despite some uncertainty as to the precise regulatory mechanisms of normal hematopoiesis,
several fundamental principles are clear. True pluripotent stem cells with self-renewal ability are rare cells, and for most progenitor cells proliferation is accompanied by lineage commitment and terminal differentiation.

HEMATOPOIESIS IN AML

Many of these fundamental principles of hematopoiesis are inoperable in AML, a disorder in which there is an accumulation of immature blasts that fail to differentiate to functional granulocytes or monocytes. Normal hematopoiesis is suppressed by either physical displacement of stem cells by leukemic cells or production of inhibitory humoral factors (such as acidic isoferritins), leading to a characteristic pancytopenia of normal blood elements.

Although AML is the result of clonal expansion of a single cell, it is clear that the resulting leukemic cell population is biologically heterogenous despite morphological homogeneity. Studies with ³H-thymidine (³H-TdR) labeling have shown that only a small fraction of AML cells are actively synthesizing DNA in vivo. This small population of proliferating leukemic cells can be identified in vitro by colony assays described below.

TECHNIQUES FOR AML COLONY GROWTH

In the initial attempts to grow leukemic colonies, normal leukocyte feeder cells were added to an agar underlayer as a source of stimulating factors, and AML blast cells were plated in a second layer on top of the underlayer. The appearance of truly leukemia derived clones in these cultures has been confirmed by cytogenetic analysis. Other colony-stimulating materials for normal CFU-GM have also been employed for growing AML clonogenic cells, eg, conditioned media from cell lines or human placental-conditioned medium (HPCM).

While these methods efficiently induce normal myeloid colony formation, they are often insufficient to permit the maximum outgrowth of AML colonies. In some cases, only single cells surviving in the dish are discerned at the end of culture or no evidence of proliferation is apparent at all. Frequently small (less than 20 cells) or somewhat larger clusters (less than 40 cells) are produced during culture. Using standard culture techniques, the nongrowing and small cluster-forming groups of patients account for the majority (60% to 70%) of the newly diagnosed cases of AML. In fact the poor responsiveness of AML-CFU to stimulation has been explained by their variable and decreased sensitivity to CSF.

By analogy with the observations that each type of normal hematopoietic progenitor cell requires a specific colony assay, attempts have been made to develop more efficient AML colony methods. Exposure of the cells to phytohemagglutinin (PHA) during a 15-hour preincubation in suspension and subsequent culturing in a soft agar dish with a leukocyte feeder allowed for the formation of significant numbers of AML colonies of more than 50 cells in the majority of patients with AML. In subsequent modifications of the technique, PHA was added directly to one-stage cultures that contained irradiated leukocytes in the agar underlayer and the AML target cells in a liquid overlay, or more recently PHA and HPCM in combination have been used as additions to agar cultures. PHA-leukocyte-conditioned medium as a stimulus in methylcellulose. Repeated feeding of the dishes during the incubation time has been suggested to enhance the colony-forming efficiency as well. These modified cultures are permissive to AML colony formation in 80% to 90% of cases and may promote growth of unique clonogenic cells. Seven-day colonies are composed of morphologically identifiable blast cells carrying cytogenetic AML markers. The blasts of colonies show partial but aberrant maturation when analyzed with combinations of immunological surface markers.

T lymphocytes and monocytes are the active components of the leukocyte feeder layer when used in combination with PHA for stimulation of AML-CFU. Natural killer cells may inhibit AML clonogenic growth in vitro. The basis of the higher AML cloning efficiency of the PHA cultures has not been elucidated. The improvement of growth under these conditions may be due to the supply of another inducer that affects AML clonogenic cells, or the release of a cofactor that enhances the colony-forming response to CSFs by causing a shift in the dose response curve. It is less likely that the enhancement of AML colony formation is due to a direct effect of PHA exerted on AML-CFU. PHA as a single ingredient in culture gives poor growth whereas the use of PHA-LCM (which would contain only minimal concentrations of PHA in the final colony cultures) results in effective stimulation.

For culture systems with PHA, growth of normal T cells must be avoided by effective T cell separation from the AML marrow/blood sample plated. T cell marker analysis of colonies is an integral part of the procedure to rule out interfering T cell clone formation. It would be important to identify the growth factors provided by PHA and leukocytes that stimulate the proliferation of AML-CFU: (a) for our understanding of the requirements of AML clonogenic cell proliferation and for delineating abnormalities thereof (eg, for growth factor receptor studies), (b) for separation of these factors from inhibitors, and (c) for standardization of culture techniques for optimal and reproducible application. As PHA-stimulated cells release a wide variety of lymphokines including hematopoietic growth factors that affect different cell types and cell functions, there may be multiple regulators of AML-CFU proliferation including both stimulatory and inhibitory factors. Studies with purified CSFs produced in vitro from cloned genes are likely to provide answers to these questions. Studies with recombinant human GM-CSF (from a gene normally expressed by T lymphocytes) indicate that this factor is a growth promoter of AML cells, but not necessarily the only such factor. Preliminary studies with recombinant human G-CSF and M-CSF indicate that G-CSF is both active in promoting AML-CFU proliferation and further is synergistic with GM-CSF, while M-CSF promotes growth in only occasional cases (Griffin J, unpublished observations) (Table 1).
**AML CLONOGENIC CELLS**

**Table 1. Effects of Recombinant Human CSFs on Growth of AML-CFU**

<table>
<thead>
<tr>
<th>CSF*</th>
<th>CFU-GEMM</th>
<th>BFU-E</th>
<th>CFU-GM</th>
<th>CFU-M</th>
<th>CFU-G</th>
<th>AML-CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CSF</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G-CSF</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Recombinant human CSFs derived from media conditioned by COS cells transfected with full length cDNAs of CSF genes, supplied by Dr Steven Clark, Genetics Institute, Cambridge, MA. CFU-GEMM, multipotent progenitor cell; BFU-E, erythroid burst forming unit; CFU-GM, CFU-M, G, granulocyte and/or monocyte colony forming unit. CFU-GEMM and BFU-E assays performed in methylcellulose, all others in agar.

**BIOLOGICAL CHARACTERISTICS OF CLONOGENIC CELLS IN AML**

**Cell cycle.** As in the case of normal human bone marrow–derived colony-forming cells, it is difficult to determine the in vivo significance of AML-CFU. However, there are several observations that suggest AML-CFU function as leukemic progenitor cells. First, it is likely that AML-CFU are among the group of in vivo cells that are actively proliferating. Thymidine suicide studies show that a very high proportion of AML-CFU are in S phase of the cell cycle and thus are synthesizing DNA in vivo. The thymidine suicide rate is variable, but in some cases is as high or higher than for normal bone marrow CFU-GM. Marie has shown that thymidine labeling index of blasts correlates with the rate of leukemic colony formation. The generation time and duration of S-phase of AML-CFU have been measured by Minden and shown to be similar to normal bone marrow CFU-GM. Thus, AML-CFU are actively cycling and may make up the majority of proliferating cells in vivo.

**Self-renewal capacity.** The second property of AML-CFU that suggests their role as progenitor cells is self-renewal capacity. Colonies can be recovered easily from methylcellulose cultures, pooled, and replated to give an estimate of secondary replating efficiency (PE2). Considerable variability in PE2 has been observed among different patients, but PE2 was stable when cells from a single patient were repeatedly tested, even at relapse. PE2 was generally <1% and rarely >10%. Tertiary replating efficiency was always low, and more than 4 generations of colonies have not been observed. The marked variation in PE2 among different patients has been interpreted by McCulloch and colleagues to be due to random determination of self-renewal versus commitment to "differentiation," which as in the case of normal progenitor cells, would be associated with loss of proliferative capacity. When secondary colonies are obtained, they tend to have the same size and morphology as the primary colony.

There is some evidence that more than one colony may be grown in some patients. True colonies often coexist with numerous clusters. Löwenberg has investigated the PE2 of large and small aggregates and found that the PE2 of large colonies is much greater than that for small colonies or clusters. However, secondary colonies were observed occasionally in the small clusters, although for the most part large secondary colonies were derived only from large primary colonies. The significance of finding large and small colonies is not clear. For normal myeloid progenitor cells, cluster-forming cells are more mature than (and are derived from) colony-forming cells, but neither form secondary colonies. It seems likely that leukemic cluster-forming cells are for the most part derived from colony-forming cells. In some cases the parental cells of colonies and clusters in AML can be distinguished by different surface markers. This may indicate that they represent subsequent differentiation stages, but this remains to be established. Overall, these observations suggest that the leukemic clone may be organized in a fashion similar to normal myelopoiesis, with a hierarchy of progenitor cells generating large numbers of "differentiated" nonproliferative leukemic cells.

**DIFFERENTIATION OF CLONOGENIC CELLS IN VITRO**

The third property of AML-CFU consistent with their role as progenitor cells is the capacity to undergo differentiation in vitro, at least to a limited extent. It is evident that most daughter cells of AML-CFU exit from the cell cycle and cannot again be induced to proliferate in vitro, a property associated with normal granulocyte/monocyte differentiation. Evidence that AML-CFU may "differentiate" during this process came initially from cytological examination of colony cells but cellular morphology was often bizarre and maturation incomplete. More recent data on the abilities of AML-CFU to differentiate in vitro comes from surface marker analysis. A number of investigators have generated monoclonal antibodies that recognize cell surface antigens expressed by normal myeloid cells. Some of these antibodies detect antigens that are expressed at discrete stages of normal progenitor cell differentiation and can be used to distinguish cells at various levels of maturation. Many of the differentiation antigens identified by these antibodies are expressed by AML cells, particularly antigens normally expressed by myeloid progenitor cells. Using immunofluorescence and flow cytometry, such monoclonal antibodies have provided the ability to "quantitate" the degree of cellular differentiation of leukemic cells and cell lines in suspension culture.

Marie studied the acquisition of the granulocyte differentiation antigen MY1 antigen during formation of blast colonies by leukemic cells from eight AML patients. In five of eight cases, the percentage of MY1+ cells in developing colonies increased over a period of two to ten days. In some, but not all, cases this correlated with increased naphthol AS-D chloroacetate esterase (CAE) activity, whereas MY1 expression always correlates well with CAE activity in normal progenitor cells. This was interpreted as showing that many components of granulopoietic differentiation occur in blast colony formation, but different components may be expressed abnormally. This study was extended by Griffin, who showed that the monocyte antigen MY4 could not be detected on AML-CFU cells prior to in vitro culture, but MY4+ cells could be readily detected in leukemic colonies after several days of culture. Similarly, Wouters and Löwenberg showed that...
AML-CFU from patients with AML lacked the granulocyte differentiation antigen B4.3, but acquired this antigen during colony formation. In an additional seven cases, increased numbers of blasts staining for B4.3 or B13.9 antigens were observed after colony formation. In most cases, differentiation was qualitatively and kinetically aberrant when compared with normal CFU-GM cells. For example, in one case expression of an unexpected marker was detected; it was the T lymphocyte differentiation antigen T6. Thus, evidence from several surface marker laboratories has shown that clonogenic cells in AML undergo an aberrant type of “differentiation” during colony formation. In most cases, the resulting cells acquire maturation-associated properties: loss of proliferative potential, loss of self-renewal capacity, and acquisition of new surface proteins that reflect cellular differentiation to a partial extent. This process occurs “spontaneously” in the presence of growth factors but it may be possible to accelerate differentiation by addition of certain differentiation-inducing chemicals such as dimethylsulfoxide, phorbol myristic acid (TPA), or gamma-interferon. These compounds are potent inducers of differentiation of leukemic cell lines such as HL-60 and some fresh leukemic cells in suspension culture, while GM-CSF is not generally an effective inducer of human leukemic cell lines. More studies are necessary to determine the effects of these agents on clonogenic cells. TPA has been shown to inhibit AML-CFU formation to a greater extent than CFU-GM formation, but this effect may be dose dependent, with very low concentrations of TPA (10^{-10} mol/L) increasing the secondary plating efficiency.

GROWTH OF AML BLASTS IN SUSPENSION CULTURE

Except in rare cases, growth of AML cells in methylcellulose or agar is self-limited and proceeds for only a short period of time. AML-CFU will also proliferate in liquid suspension culture for periods of up to 70 days when stimulated by PHA-LCM, and can be maintained in exponential growth for periods of several weeks. The addition of methylcellulose to liquid cultures inhibited growth. High cell density was required for maximum growth, but this requirement could be replaced by adding blast cell membranes (5 x 10^6 cell equivalents/mL). The membrane-bound factor has not yet been further characterized.

AML cells have also been grown in long-term marrow cultures, both with and without preformed normal adherent layers. In some cases, but not all, the long-term culture conditions appeared to favor growth of normal progenitor cells rather than AML-CFU. In many cases, adherent layers failed to develop from AML marrows. When AML cells were added to preformed adherent layers, AML-CFU proliferated very briefly and the leukemic cells tended to differentiate rapidly.

SURFACE PHENOTYPE OF CLONOGENIC CELLS IN AML

Recently, considerable progress has been made in the identification of normal progenitor cell surface antigens, and different stages of normal progenitor cells can now be readily distinguished by their phenotypes. These progenitor cell antibodies offer the possibility of identifying a normal counterpart cell for AML-CFU. Three studies have been reported in which AML-CFU have been analyzed with panels of monoclonal antiprogenitor cell antibodies (Fig 1) by complement lysis. Lange et al studied ten cases with S-13, S-8,6, HLA-DR, S16-144, S4.7, and R1B19. Sabbath et al studied 20 cases with HLA-DR, MY9, PM-81, AML-2-23, Mol, Mo2, and MY4. Lowenberg studied seven cases for expression of S3-13, S4-7, S8.6, S16-144, S16-109, S17-25, B4.3, and HLA-DR antigens. The results of these studies are summarized in Table 2. Of the 37 cases tested, AML-CFU from all cases expressed one or more of the antigens tested. In most cases, antigens were expressed either by >90% of AML-CFU or by <10% of AML-CFU. However, some antigens, particularly those associated with more mature cells, were expressed heterogeneously by AML-CFU from an individual patient, being found on some, but not all, clonogenic cells. This finding suggests that multiple populations of AML-CFU may exist in some cases. However, low antigen density may account for some of this variability. Unfortunately, no CFU-GEMM-specific antigens have been identified, and all of the CFU-GEMM reactive antibodies employed in these studies also react with more mature progenitor cells. Nevertheless, several antibodies are available, such as PM-81, AML-2-23, and S4-7, which react with intermediate-level progenitor cells, but not with CFU-GEMM. Therefore, reactivity of one of these antibodies with AML-CFU identifies these cells as being the leukemic counterpart of a normal cell at least as mature as the earliest normal progenitor cell expressing that antigen. Thus, 65% and 69% of AML cases had a dominant AML-CFU population that expressed the day 14 CFU-GM antigens PM-81 and S4.7, respectively. Thirty per cent of cases had AML-CFU that expressed the day 7 CFU-GM antigen AML-2-23. Interestingly, 8 of 10 cases studied by Lange et al expressed the late CFU-GM antigen R1B19. Antigens expressed by normal cells only at the blast stage or later (Mol, B4.3, MY3) were not detected on AML-CFU (Table 2). These results suggest that AML-CFU phenotypes...
AML CLONOGENIC CELLS

Table 2. Surface Antigens of Leukemic Clonogenic Cells

<table>
<thead>
<tr>
<th>Antibody or Antigen</th>
<th>Least Mature Normal Precursor Cell* Expressing Antigen</th>
<th>Number of AML Cases With &gt;50% Antigen Positive L-CFC Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3-13</td>
<td>CFU-GEMM</td>
<td>17/17 (100%)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>CFU-GEMM</td>
<td>33/37 (89)</td>
</tr>
<tr>
<td>S8-6</td>
<td>CFU-GEMM</td>
<td>11/16 (69)</td>
</tr>
<tr>
<td>MY9</td>
<td>CFU-GEMM</td>
<td>19/20 (95)</td>
</tr>
<tr>
<td>S16-144</td>
<td>CFU-GEMM</td>
<td>16/16 (100)</td>
</tr>
<tr>
<td>PM-81</td>
<td>day 14 CFU-GM</td>
<td>13/20 (65)</td>
</tr>
<tr>
<td>S4.7</td>
<td>day 14 CFU-GM</td>
<td>11/16 (69)</td>
</tr>
<tr>
<td>R1B19</td>
<td>day 7 CFU-GM</td>
<td>8/10 (80)</td>
</tr>
<tr>
<td>AML-2-23</td>
<td>day 7 CFU-GM</td>
<td>6/20 (30)</td>
</tr>
<tr>
<td>Mo1</td>
<td>blast/Promyelocyte</td>
<td>1/20 (5)</td>
</tr>
<tr>
<td>B4.3</td>
<td>metamyelocyte</td>
<td>0/7 (0)</td>
</tr>
<tr>
<td>MY3</td>
<td>monocytes</td>
<td>0/20 (0)</td>
</tr>
</tbody>
</table>

*CFU-GEMM, granulocyte/erythrocyte/monocyte/megakaryocyte colony-forming cell; CFU-GM, granulocyte/monocyte colony-forming cell.

varies among different patients. Overall, about 1/3 of cases have had AML-CFU phenotypes comparable to CFU-GEMM (or earlier) cells, while the other 2/3 have expressed phenotypes of committed progenitor cells (day 7 or day 14 CFU-GM). This information is summarized schematically in Fig 2.

Abnormal differentiation in AML is reflected in the considerable variability of AML-CFU phenotypes and in the presence of phenotypes not found in normal cells. Not only the presence or absence of certain determinants but also the density at which these markers are expressed on the clonogenic cells have served to characterize abnormal classes of phenotypes, and this could be useful for diagnostic purposes in the future.

COMPARISONS OF SURFACE PHENOTYPES OF AML-CFU WITH THOSE OF AML BLASTS

The phenotypes of the clonogenic cells have also been compared to the phenotypes of the majority of circulating leukemic cells. In the 10 cases studied by Lange, very “early” progenitor cell antigens such as S3-13 and S8-6 were universally expressed by AML-CFU, but were generally lost by the rest of the population in each case. Similarly, in the 20 cases studied by Sabbath, early progenitor cell antigens such as HLA-DR and MY9 were expressed on a significantly higher fraction of AML-CFU than of the total leukemic cell population. In addition, several “late”-appearing antigens, Mo1, Mo2, and MY3, were not expressed by AML-CFU, but were commonly expressed on the total population (9 of 20 cases had greater than 50% of leukemic cells expressing one or more of these antigens). In fact, in each of these 20 cases, the total cell population expressed new differentiation antigens lacking from the less mature AML-CFU population. These results demonstrate that differentiation, although incomplete and often markedly aberrant, is likely to be an ongoing event in vivo in most cases of AML.

The major limitation of this type of analysis is the possibility that the AML-CFU proliferating in vitro is not the most immature leukemic stem cell, but a derivative of a stem cell that does not grow well with available culture systems. Using surface marker analysis to identify different stages of AML-CFU, it should now be possible to optimize culture conditions for growth of the earliest AML-CFU.

THE CELL OF ORIGIN OF AML

Surface marker analysis has shown that AML clonogenic cells may arise at multiple points in hematopoiesis in different patients, and are less differentiated than their progeny in vitro and possibly in vivo. If AML-CFU are representative of the ancestral cell that gave rise to the leukemic clone, then these observations may shed some light on the cell of origin of AML. Unlike chronic myeloid leukemia where genetic and glucose-6-phosphate dehydrogenase (G6PD) isoenzyme studies have demon-
Strated that the cell of origin is a pluripotent stem cell in all cases, similar studies in AML have suggested that some cases arise at the level of pluripotent stem cells while others arise at much later stages. Involvement of the erythroid lineage as well as the granulocyte lineages may be particularly common in cases with a preceding myelodysplastic syndrome. One of the earliest demonstrations of involvement of the erythroid lineage in AML was by Blackstock who described a case in which a chromosomal marker was detected in bone marrow cells that were identified as erythroid by their incorporation of radioactive iron. Even more compelling evidence comes from glucose-6-phosphate dehydrogenase (G6PD) studies. In 1981, Fialkow reported four cases of AML in which two young G6PD heterozygous patients, ages 10 and 11, expressed only a single isoenzyme in leukemic cells, but both isoenzymes in erythrocytes at diagnosis and remission. In contrast, two older patients, ages 69 and 70, had only a single isoenzyme in leukemic cells, erythrocytes, and platelets at diagnosis. In one patient who went into remission, both isoenzymes were later found in erythrocytes, granulocytes, and platelets. In another adult case, analysis of B lymphoid cell lines suggested involvement of the lymphocyte stem cell as well. It has been postulated that the shorter survival of adults compared to children with AML may be due to the more frequent pan-myeloid nature of the illness in adults. G6PD heterozygotes with AML are rare, however, and only a small number of cases have been studied.

One problem of G6PD analysis is that it may underestimate the frequency of involvement of multipotent stem cells. If the leukemic stem cells are limited in either the extent to which they may undergo erythroid differentiation, or in their ability to complete erythroid differentiation, then these cases would be difficult to detect. Considering the profound abnormalities of myeloid differentiation in AML, abnormal erythroid differentiation would not be unexpected. If the leukemic clone supplies 10% of erythrocytes, the ratio of isoenzymes would theoretically be 1.22:1, while 30% are supplied, the ratio would be 1.86:1. Thus, the contribution of the leukemic clone to the erythron must be substantial for detection by isoenzyme analysis, as ratios as high as 1.5:1 or higher can be seen in normal tissues.

The finding from surface marker analysis that ⅚ to ⅓ of AML-CFU have phenotypes of cells committed to granulocyte/monocyte differentiation in agreement with the results of isoenzyme studies that many cases of AML arise at the level of the CFU-GM. The correlation between the two methods is not known, however, and it would be valuable to phenotype AML-CFU from patients heterozygous for G6PD.

**PROGNOSTIC VALUE OF AML-CFU**

Several investigators have attempted to correlate different patterns of leukemic colony growth with prognosis. As noted previously, the use of a leukocyte feeder layer often generates only scant numbers of colonies, and thus the recovery to normal colony numbers may be used as an indicator of ensuing hematological remission while persistent leukemic growth indicates induction failure. Remission colonies exhibit the nonleukemic karyotype. Not only the incidence and karyotype of clone forming cells, but also their buoyant density, thymidine suicide index (as a measure of active cell cycle status of normal colony-forming units), and morphological maturation during colony growth all revert to normal following attainment of complete remission. Recurrence of abnormal growth pattern following attainment of complete remission usually heralds AML relapse.

Various categories of AML colony and cluster growth have been defined in order to test for prognostic significance: (a) no formation of colonies or clusters in culture, (b) mainly small clusters, (c) mainly large clusters, (d) colonies are formed but are rare and exist in the presence of a normal colony-cluster ratio, or (e) colonies coexist with numerous clusters (high cluster-colony ratio). In one large study the categories a, c, and e were associated with a low rate of complete remission. However, in this study diverse chemotherapy programs (often without an anthracycline) were used for remission induction, resulting in an overall complete remission rate of only 36%. Two later studies revealed a poorer prognosis for patients belonging to the large cluster-forming category as compared to the more favorable non-growing and small cluster-forming patient groups (Table 1).

In another study an attempt has been made to relate the apparent diminished sensitivity of AML-CFU to CSF to the probability of remission induction success. It was suggested that in patients in whom the clone-forming cells exhibited a higher threshold for CSF, the remission rate was reduced. As yet, little information has been collected on the prognostic value of PHA-containing cultures. One study of 19 patients has indicated that the self-renewal capacities of AML-CFU were predictive of a favorable treatment result. Low secondary plating efficiency, assessed by recloning primary colonies in secondary cultures, correlated positively with successful induction. As the abnormal colony-cluster pattern characteristic of patients with AML is frequently expressed in patients with the myelodysplastic syndromes, these abnormalities do not serve as a convenient parameter for assessing progression of these diseases toward AML. The newer PHA supplemented assay has been evaluated in a small group of patients with myelodysplastic syndromes and did not provide prognostic information.

**Table 3. Pattern of Colony/Cluster Growth in Vitro in Relation to Remission Induction Rate in AML**

<table>
<thead>
<tr>
<th>Colony Morphology*</th>
<th>No Growth</th>
<th>Small Aggregates</th>
<th>Large Aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study (Reference)</td>
<td>No. of Pts.</td>
<td>% CR</td>
<td>No. of Pts.</td>
</tr>
<tr>
<td>Moore et al12</td>
<td>13</td>
<td>15</td>
<td>51</td>
</tr>
<tr>
<td>Spitzer et al13</td>
<td>21</td>
<td>76</td>
<td>36</td>
</tr>
<tr>
<td>Browman et al14</td>
<td>12</td>
<td>83</td>
<td>16</td>
</tr>
</tbody>
</table>

*Small aggregates, clusters of 20 cells or less; large aggregates clusters of more than 20 cells. For the purpose of intercomparison of the three studies, the subdivision of growth patterns was modified according to identical criteria.

†CR, complete remission.
SENSITIVITY OF AML-CFU TO CHEMOTHERAPY IN VITRO

Based on the assumption that the clone-forming cells are primarily responsible for perpetual growth, several investigators have attempted to correlate the in vitro sensitivity of AML-CFU to different cytostatic agents with the clinical efficacy of the drugs in vivo.

In pilot studies with anthracyclines, it has been shown that maximal cell kill of AML-CFU is achieved within approximately ten minutes. More prolonged exposures or the continuous addition of the drug to the colony dishes did not add to the cytoreductive effect. Results are identical when fresh or cryopreserved blasts are used.

Cytosine arabinoside and (hydroxy)daunomycin, common drugs in current remission induction schedules, have been evaluated most frequently. The results of the compiled studies are summarized in Table 4. In spite of the methodological variations, it is of note that the correlations between the in vitro sensitivity of AML-CFU to Ara-C and anthracyclines and the ability of these drugs to induce complete remissions are positive in most studies. It is possible that the combined information from parallel sensitivity testing for single drugs or from exposure of the cells to multiple drug mixtures permits the most reliable predictions. However, there is substantial overlap in the range of cell kill of AML-CFU between sensitive and resistant leukemias, and absolute discrimination between responders and nonresponders is not possible. The method has been employed to investigate the relative efficacy of certain new anthracycline analogues as well, ie, (hydroxy)daunomycin, tetrapyranyl-doxorubicin, and aclacinomycin. The findings of these studies provided evidence for non-cross resistance among these agents. This system has also been used to evaluate differentiation-inducing agents such as interferon and retinoic acid.

At present in vitro sensitivity testing of AML-CFU has not become generally accepted for several reasons. The assay requires a minimum of seven days of culture, which is a drawback for clinical use. The methods employed in various studies are often quite different, so that direct comparisons between the results cannot be made. For example, the susceptibility of AML-CFU to cytosine arabinoside has been determined in some cases by adding the drug to colony cultures (continuous exposure), but in other studies the cells have been exposed during a short incubation to the drug followed by measuring AML-CFU survival in a colony test (preincubation). The levels for distinguishing in vitro sensitivity from in vitro resistance of AML-CFU (% cytocidal) vary greatly among different studies, and within one study different cut-off points have been considered for individual agents. Colony techniques and the way of evaluation (colony size and day of culture) in reported studies commonly are different. The choice of the schedules of remission induction chemotherapy and the selection of patients (eg, untreated versus relapsed AML) have been diverse. If one also considers the small numbers of patients included in most of these investigations, that factors other than chemotherapy

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Table 4. Reported Studies on Correlations Between in Vitro Sensitivity of AML-CFU to Chemotherapeutic Drugs and in Vivo Response

<table>
<thead>
<tr>
<th>Chemotherapy</th>
<th>Ref 116†</th>
<th>Ref 113</th>
<th>Ref 58</th>
<th>Ref 48</th>
<th>Ref 111</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ara-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation dose</td>
<td>0.3 ug/mL</td>
<td>—</td>
<td>10^-5 mol/L</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Incubation time</td>
<td>1 hr</td>
<td>—</td>
<td>continuous§</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>% Loss of AML-CFU</td>
<td>&gt;30% &lt;30%</td>
<td>—</td>
<td>&gt;50% &lt;30%</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Patients (n)</td>
<td>6 9</td>
<td>—</td>
<td>10 12</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pts. with CR (n)</td>
<td>5 2</td>
<td>—</td>
<td>9 3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(P = 0.04)‡</td>
<td>(P &lt; 0.003)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthracyclines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation dose</td>
<td>0.1 ug/mL DNR</td>
<td>—</td>
<td>10^-5 mol/L</td>
<td>—</td>
<td>1 ug/mL HDNR</td>
</tr>
<tr>
<td>Incubation time</td>
<td>1 hr</td>
<td>—</td>
<td>10 min</td>
<td>—</td>
<td>1 hr</td>
</tr>
<tr>
<td>% Loss of AML-CFU</td>
<td>&gt;30% &lt;30%</td>
<td>—</td>
<td>&gt;90% &lt;90%</td>
<td>—</td>
<td>&gt;90% &lt;90%</td>
</tr>
<tr>
<td>Patients (n)</td>
<td>9 5</td>
<td>—</td>
<td>13 8</td>
<td>—</td>
<td>10 13</td>
</tr>
<tr>
<td>Pts. with CR (n)</td>
<td>7 1</td>
<td>—</td>
<td>8 3</td>
<td>—</td>
<td>7 0</td>
</tr>
<tr>
<td>(P = 0.09)‡</td>
<td>(P = 0.39)</td>
<td>(P &lt; 0.001)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined drugs</td>
<td>Ara-C + DNR</td>
<td>Anthracycline + VCR + Ara-C</td>
<td>—</td>
<td>Ara-C + Anthracycline + Predn</td>
<td></td>
</tr>
<tr>
<td>Incubation time</td>
<td>1 hr</td>
<td>—</td>
<td>1 hr</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>% Loss of AML-CFU</td>
<td>&gt;20% &lt;20%</td>
<td>SI high</td>
<td>SI low</td>
<td>—</td>
<td>&gt;30% &lt;30%</td>
</tr>
<tr>
<td>Patients (n)</td>
<td>9 5</td>
<td>5 9</td>
<td>15 23</td>
<td>—</td>
<td>11 7</td>
</tr>
<tr>
<td>Pts. with CR (n)</td>
<td>8 1</td>
<td>4 1</td>
<td>—</td>
<td>(P = 0.02)</td>
<td>(P = 0.02)</td>
</tr>
</tbody>
</table>

*aAra-C, cytosine arabinoside; DNR, daunorubicin; HDNR, hydroxydaunorubicin; VCR, vincristin; Predn, prednisone; CR, complete remission.
†Two remission induction failures as a result of hypoplastic death are not included.
‡Statistical significance is only indicated for studies involving 15 patients or more; NS, not significant. P values determined by Fiser exact test.
§Continuous, the drug was added to the colony assays during the entire culture period.
|| SI or sensitivity index refers to the relative sensitivity of AML-CFU and indicates the ratio of % survival of CFU-GM v % survival of AML-CFU following drug exposure. High SI, more than 1.0; Low SI, less than 1.0.
sensitivity of AML-CFU determine whether a patient will survive remission induction treatment (eg, age), and that all these studies were retrospective, it is clear that the value of testing the chemotherapy sensitivity of AML-CFU is far from definitely established.

CONCLUSIONS

Clonogenic cells in AML have offered a unique opportunity to investigate the concept of stem cells in hematopoietic malignancies. AML-CFU have properties of true stem cells, including high rate of DNA synthesis in vivo, high proliferative capacity in vitro, self-renewal capacity, and ability to differentiate to nonproliferative cells. Surface antigen studies show that AML-CFU are heterogeneous among different patients, and are derived from normal counterpart cells at various stages of myeloid differentiation. It may be important to determine the growth factors required for AML-CFU proliferation to optimize in vitro culture, to determine if even earlier stem cells exist, and to better understand how these cells have obtained an apparent proliferation advantage over their normal cellular counterparts. Finally, AML-CFU assays may provide prognostic information through the assessment of self-renewal capacity and determining sensitivity to chemotherapeutic agents. Further investigation in this area is warranted.

REFERENCES

AML CLONOGENIC CELLS


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