Murine Erythroid Cell Lines Derived with c-myc Retroviruses Respond to Leukemia-inhibitory Factor, Erythropoietin, and Interleukin 3

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Abstract
The transforming potential of the c-myc gene is shown here, for the first time, to include murine erythroid cells. Continuously growing cell lines were reproducibly generated by infection of day 13 CBA fetal liver cells with novel recombinant c-myc retroviruses. By cytostaining, most cells resembled early erythroblasts, but certain lines also contained significant numbers of hemoglobinized cells. RNA analysis revealed substantial expression of the genes encoding β-globin and the erythroid-specific transcription factor GF-1. Although apparently immortal, the lines were not initially transplantable. Thus, constitutive myc expression in early erythroid cells can enhance their self-renewal capacity but is insufficient to fully transform them.

The cell lines proliferated without the addition of exogenous factors, but their clonogenicity in semisolid medium was enhanced in the presence of erythropoietin, interleukin 3, or leukemia-inhibitory factor. In combination with either interleukin 3 or erythropoietin, leukemia-inhibitory factor also facilitated differentiation of certain lines. These results suggest that leukemia-inhibitory factor may have a previously unsuspected role in the regulation of erythropoiesis and could be considered as a possible therapeutic agent for the clinical management of erythroleukemia.

Introduction
The c-myc gene is expressed by proliferating cells of most lineages and encodes a nuclear protein with structural motifs (helix-loop-helix, leucine zipper) reminiscent of a transcription factor (1). Although the normal function of c-myc is still unknown, its deregulated expression has been implicated as a key event in the evolution of many hemopoietic neoplasias, primarily lymphoid. In Burkitt lymphomas and rodent plasma cell tumors, for example, c-myc is translocated to an immunoglobulin gene locus (2), and the consequent subjugation of myc expression to immunoglobulin enhancers has been shown to be highly leukemogenic in transgenic mice (3). Infection of animals and birds with myc-bearing retroviruses primarily causes myeloid disease (4–6), and myeloid cell lines have been the major outcomes of in vitro infection of hemopoietic cells (7–10). No spontaneous erythroid tumors have involved c-myc, nor have the erythroleukemias that are induced in mice by Friend virus infection (11). However, tumorigenic erythroid cell lines have been produced in vitro by infection of fetal liver cell lines with a retrovirus (12) expressing both v-ral and v-myc (12).

We report here that erythroid cell lines can be reproducibly generated by infection of murine fetal liver cells with retroviruses expressing murine c-myc. None of the continuously growing cell lines was initially transplantable, although slowly growing tumors were obtained from cultures which had been passage in vitro for several months. Thus, enforced myc expression potentiates the immortalization of erythroid precursors but does not fully transform them. Similar conclusions have previously been reached for primary fibroblasts (13–16).

The clonogenicity of the myc-induced erythroid lines in semisolid media was enhanced in response to IL-3, Epo, and, surprisingly, LIF, a factor purified and cloned (17) on the basis of its capacity to induce terminal differentiation of certain myeloid cell lines (18). In combination with either IL-3 or Epo, LIF also enhanced the capacity of cells of certain lines to mature to the hemoglobinized (benzidine-positive) stage. LIF is a remarkable multifunctional regulator, with striking actions on a diverse range of cell types, but so far it has not been demonstrated to be a major proliferative factor for hemopoietic cells (19). Acting alone, it has no apparent proliferative effects on normal fetal or adult hemopoietic cells (20), but, when combined with IL-3, it enhances the proliferation of blast colonies (21) and megakaryocytic colonies (5). It is also known to be a proliferative stimulus for the continuous hemopoietic cell line DA-1 (22). The present observations suggest that LIF may have a previously unsuspected role in regulating normal erythroid development.

Results
Novel Erythroid Cell Lines Derived by c-myc Virus Infection of Fetal Liver. Two new helper virus-free retroviruses, Zen[myc] and MPZen[myc] (Fig. 1), were used to explore the effect of constitutive myc expression in im-
mature hemopoietic cells. Cells infected with the Zen virus express c-myc under the control of a Moloney virus LTR, whereas those harboring a MPZen provirus utilize a LTR derived from the myeloproliferative sarcoma virus (23). CBA fetal liver (day 13) cells were cocultivated for 2 days with irradiated virus-producing \( \Phi \) fibroblasts in the presence of a source of IL-3. Nonadherent fetal liver cells were then harvested from the monolayers and thereafter maintained continuously in liquid culture, either with or without exogenous IL-3. Most cells died within the first week, but characteristic grape-like clusters of cells became apparent after a second week, even in cultures lacking added IL-3. The clusters were often tethered by long processes to the bottom of the culture vessel (Fig. 2A) and, in undisturbed cultures, grew very large, containing many thousands of cells. No such clusters were observed in control cultures mock infected by cocultivation with the parental \( \Phi \) line, although, if IL-3 stimulated, these cultures produced transient populations of mast cells. The nature of the tethering cells is not known, but they may correspond to the "clasmatosing macrophages" noted previously in association with hemopoietic cells in cultures of explants of fetal liver (Fig. 13 in Ref. 24). They were apparently lost from the cultures after several passages but may have actively contributed to their establishment by, for example, providing growth factor(s).

Twelve independent cell lines were derived in this fashion, and, for five of them, clones were subsequently obtained by expansion of colonies grown in methylcellulose (see below) (Table 1). A striking feature of most lines, even after cloning, was the marked heterogeneity in the size and shape of the cells, and the viability of the cultures was rarely >70%. Most lines continued to exhibit markedly clumped growth (e.g., ME17.EB in Fig. 2B), but a few (e.g., ME15.C2 in Fig. 2C) grew primarily as dispersed cells and small clusters.

The cellular origin of the lines was not immediately apparent. May-Grünwald-Giemsa staining revealed an intensely basophilic cytoplasm (Fig. 3, A and B) and a large perinuclear vacuole, presumably the Golgi zone. The plasma membrane was often ruffled, multinucleate cells were common, and mitotic figures were prominent. The cells did not appear to be B lymphoid, because they did not stain for the B lymphoid marker Ly5 (B220), and their DNA lacked any IgH rearrangement (data not shown). Neither T lymphoid (Thy-1) nor myeloid (Mac-1) surface antigens could be detected by immunostaining, but high autofluorescence precluded any firm conclusions about phenotype.

The brownish color of cell pellets prepared from certain lines suggested that erythroid cells might be present. We therefore screened by Northern blot analysis for \( \beta \)-globin expression (Fig. 4A). All lines proved to contain globin mRNA, a definitive marker for the erythroid lineage (Table 1). Four lines also stained with an antoglobin antibody, but the frequency of positive cells ranged from only 3% (ME16) to ~70% (ME15), suggesting that the lines varied in their capacity for maturation. Of two clonal lines analyzed, one (ME15.C2) stained weakly with the TER119 antibody, which reacts with erythroid cells and day 14-16 fetal liver cells (25). The lines were subsequently analyzed for expression of other genes impli-
Table 1. Mouse fetal erythroid lines derived by infection with a c-myc virus.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Clonality</th>
<th>Gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zen[myc]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME1</td>
<td>Mono[2]</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME2</td>
<td>Mono[2]</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME2.111</td>
<td>Mono[2]</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME4</td>
<td>Oligo</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME5</td>
<td>Oligo</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>MPZen[myc]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME11</td>
<td>Oligo</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME12</td>
<td>Oligo</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME13</td>
<td>Oligo</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME14</td>
<td>Mono[5]</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME14.4'</td>
<td>Mono[5]</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME15</td>
<td>Oligo</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME15.A6</td>
<td>Mono[2]</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME15.C2</td>
<td>Mono[2]</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME16</td>
<td>Mono[1]</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME16.E1</td>
<td>Mono[1]</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME17</td>
<td>Oligo</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME17.E2</td>
<td>Mono[1]</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ME17.E8</td>
<td>Mono[5]</td>
<td>+ (low) + + + +</td>
</tr>
</tbody>
</table>

*Cell lines derived by infection of day 13 CBA fetal liver cells with either Zen[myc] or MPZen[myc] virus (see “Materials and Methods”) were grown continuously in liquid culture. Clones indicated as ME14.4', etc., were derived from the parental lines by expanding colonies grown in methylcellulose.

*Clonality as assessed by c-myc provirus insertion pattern obtained by Southern blots of EcoR1-digested DNA. Numbers in brackets, number of proviral inserts.

The average number of cells in the colonies also increased 2-8-fold in the presence of these factors, IL-3 being most potent (Table 2), but no synergy was noted when the factors were added in pairwise combinations. With the ME15.C2 line, the optimal response was obtained with LIF and IL-3 in combination, even though IL-3 alone had no demonstrable effect (Fig. 5B). Neither line responded to G-CSF, GM-CSF, M-CSF, or IL-6, acting either alone or in combination.

**Induced Differentiation.** To investigate whether IL-3, LIF, or Epo had any effect on the differentiation of the cell lines, the proportion of colonies that contained benzidine-positive cells was determined after varying periods in agar culture. Whereas essentially no differentiation of the ME17.E8 line could be detected in the absence of factors, significant differentiation was apparent after 5 days of growth in IL-3, Epo, or LIF (Figs. 3C and 6A). After 8 days, the number of benzidine-positive colonies declined, presumably due to the death of the mature cells. Neither the cloning efficiency nor the differentiation of ME17.E8 was affected by the inclusion in the agar of 1% DMSO, which promotes differentiation of Friend virus-induced erythroid lines (11).

The ME15.C2 line appeared to be less responsive to differentiation signals than ME17.E8, since very few benzidine-positive colonies were obtained in agar containing erythropoietin, LIF, IL-3, or erythropoietin plus LIF. Inclusion of 1% DMSO in the medium reduced its cloning efficiency about 4-fold (data not shown) and enhanced...
the differentiation response, significant numbers of colonies being detected in cultures containing Epo and LIF but not IL-3 (Fig. 6B). The presence of both Epo and LIF was particularly effective, 40% of the colonies being hemoglobinized after 5 days. The cloning efficiency in DMSO was also maximal with this factor combination.

Curiously, benzidine-positive cells could not be induced in either cell line in liquid culture. The lack of differentiation seems unlikely to be due to the absence of an agar-derived factor, since inclusion of an agar underlayer in the cultures had no effect. Instead, differentiation may require efficient cell-cell contact.

**c-myc Virus-derived Erythroid Lines Are Relatively Benign.** Since many of the myc virus-infected erythroid lines were maintained continuously in culture for at least 9 months, constitutive myc expression appeared to have immortalized the cells. Transplantation tests were performed to assess whether they were malignant (Table 3). Seven of the primary lines and one clone were tested as soon as possible after establishment in culture (2-4 weeks). None of the recipient nude mice developed tumors over an observation period of 18-29 weeks.

Three cloned lines which had been maintained considerably longer in culture prior to transplantation (12-14 weeks) did produce tumors in normal syngeneic recipients. Tumor onset was moderately fast (16-18 weeks) in mice that received injections of the ME15.C2 line. With the other two lines, ME14.C4 and ME17.E8, most mice remained healthy for 28-75 weeks, even though small tumor masses were often palpable about 14 weeks earlier. One exceptional mouse that received an injection of ME17.E8 cells developed a tumor in 12 weeks. The tumor contained the same five proviral inserts as the parental cell line but also harbored an additional myc provirus. A cell line derived from that tumor, denoted ME17.EBT, had significantly different growth properties in vitro from those of the parental line; the cells were homogeneous in size, grew as single cells rather than clumps, and, significantly, were no longer responsive to LIF, Epo, or IL-3, even though the Epo receptor gene was still transcribed (Table 1).

Most tumor foci developing in transplanted mice comprised tightly packed aggregates of large immature cells with prominent mitotic activity but no evidence of morphological differentiation. In certain areas, however, some of the tumor cells were multinucleate and resembled megakaryocytes. Cloned cell lines derived from such tumors have subsequently been shown to comprise megakaryocytes and their precursors in addition to erythroid cells. Typically, the tumors developed preferentially in the lymph nodes and grossly enlarged them. In some mice, the spleen was similarly involved, but in others, it merely exhibited nonspecific hyperplasia. Tumor infiltrates were sometimes present in the liver, lung, kidney, and bone marrow but, except in the liver, were usually not extensive. Where tested, the tumor cells grew more rapidly in secondary recipients (Table 3), suggesting that selection was occurring in vivo for a more aggressive genotype.

These observations suggest that additional mutations are required for myc erythroid lines to evolve into ag-
progressive tumors. Several of the cell lines and tumors were therefore analyzed for alterations of p53, a gene frequently silenced or mutated in Friend virus-induced erythroleukemia (32). All produced abundant transcripts of normal sized p53 mRNA (Table 1), and no rearrangements were detected by Southern blot analysis of EcoRI digests (data not shown). These tests do not, however, rule out more subtle mutations. Several lines were also tested for expression of spi-1, a transcription factor gene which is normally silent in erythroid cells but is frequently activated by Friend virus in Friend erythroleukemia (33). Only 1 of 7 lines tested had detectable spi-1 RNA (Table 1), and this line was not tumorigenic. Thus, spi-1 does not appear to be involved frequently in the evolution of myc-induced erythroid tumors.

Discussion

Although the oncogenic potential of myc for the lymphoid and myeloid lineages of the hemopoietic system has been clearly documented (1–10), virtually nothing has been known of its capacity to transform erythroid cells. Indeed, a wide range of oncogenes generated erythroid colonies of limited growth potential in vitro (34, 35), but neither v-myc nor c-myc was able to do so (35). We have shown here that erythroid cell lines can be generated reproducibly in vitro from day 13 fetal liver cells by infecting them with a helper virus-free c-myc retrovirus. The erythroid character of the c-myc virus-generated fetal liver cell lines was apparent from their characteristically strong basophilic staining (Fig. 3) and their expression of the erythroid-specific genes ß-globin, GF-1, and the Epo receptor (Fig. 4; Table 1). The predominant cell type resembled an erythroblast, but more mature erythroid cells, and sometimes megakaryocytes, were also evident. The target cells for transformation presumably therefore include erythroid and erythroid/megakaryocytic progenitor cells.

The Zen retroviral vector (23) probably contributed to the success of these experiments, since more myc protein is expressed by cells infected with Zen[myc] viruses than by cells infected with c-myc retroviral vectors of the previous generation (36). The infection protocol used may well also have contributed, however, since we have also been able to generate erythroid cell lines from fetal liver with a bcr-abl retrovirus (37). The Zen vectors have also been highly effective for in vivo expression of a range of growth factors (38–41).

No transcripts of the endogenous c-myc alleles were detectable in the presence of viral c-myc expression in the infected cells (Fig. 4). Suppression of the endogenous c-myc gene by a deregulated homologue has also been observed in tumors harboring c-myc translocations, in lymphoid tumors from transgenic mice bearing Eμ-myc, Eμ-N-myc, or Eμ-L-myc transgenes, and frequently, but not always, in cells infected with other myc retroviruses (reviewed in Ref. 42). These results support the hypothesis that the c-myc gene is normally subject to negative feedback regulation, its transcription being turned off (directly or indirectly) once c-myc polypeptides exceed a critical threshold (42).

Prior to these experiments, the Friend virus complex was the only agent known to consistently transform erythroid cells (11). In Friend virus erythroleukemia, malignancy is acquired by multiple steps and involves both insertional activation of cellular oncogenes by the helper virus (32, 33) and surrogate Epo activity by the env protein of the accompanying spleen focus-forming virus (43). Similarly, the c-myc virus-infected erythroid lines were apparently not initially tumorigenic and, even though transplantable cells emerged after prolonged culture in vitro, the resulting tumors were usually very slow growing (Table 3). We infer from these results that a myc oncogene can facilitate immortalization of erythroid cells but does not completely transform them. Neither spi-1 nor p53, two genes frequently implicated in Friend erythroleukemias (32, 33), appears to play a major role in myc-induced erythroid transformation.

Constitutive myc expression within the B lymphoid compartment of transgenic mice is associated with increased cell cycle activity and an excess of immature (pre-B) cells (44). These observations led to the proposal that a deregulated myc gene enhances the probability of self-regenerative divisions at the expense of differentiation (3, 44). Consistent with this hypothesis, enforced myc expression in Friend virus-induced erythroid lines inhibited their capacity to differentiate in vitro (45–48). As expected, most cells in the c-myc virus-induced cell lines were relatively immature (Fig. 3). Differentiation was not completely blocked, however, because several lines exhibited some benzidine-positive cells when cultured in semisolid media, and their proportion could be increased by IL-3, LIF, and Epo (Fig. 6). The myc erythroid lines were apparently factor independent, whether or not they had been generated in the presence of IL-3. However, the cells were initially main-

### Table 2: Increased size of ME17.E8 colonies in the presence of growth factors

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Saline</th>
<th>LIF</th>
<th>Epo</th>
<th>IL-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>342</td>
<td>990</td>
<td>1080</td>
<td>2512</td>
</tr>
<tr>
<td>2</td>
<td>262</td>
<td>634</td>
<td>881</td>
<td>2220</td>
</tr>
<tr>
<td>3</td>
<td>214</td>
<td>1320</td>
<td>1356</td>
<td>2181</td>
</tr>
<tr>
<td>4</td>
<td>311</td>
<td>732</td>
<td>1568</td>
<td>2618</td>
</tr>
</tbody>
</table>

Mean ± SD: 282 ± 56, 919 ± 307, 1221 ± 302, 2383 ± 215.
tained at high density, and they might produce factor(s)
estential for their survival and proliferation. In this regard,
Northern blot and PCR analysis revealed low levels of G-
CSF RNA in all lines analyzed, but no detectable IL-3, IL-
6, GM-CSF, LIF, IL-5, or Epo mRNA (data not shown).
Cell lines constitutively expressing c-myc have been
shown previously to retain their need for growth factors
(8, 9, 48), although at reduced levels (48).

The clonogenicity of primary myc erythroid lines was
considerably enhanced in the presence of IL-3 and/or
LIF. Analysis of two cloned lines showed that both the
number and size of colonies growing in semisolid cultures
were increased in the presence of Epo, IL-3, or LIF (Fig.
5; Table 2). The enhanced proliferation with IL-3 and Epo
was not unexpected, since both are known proliferative
stimuli for early erythroid precursors, including those of
fetal origin (49, 50). However, the enhanced proliferation
and differentiation (Fig. 6) to hemoglobin-containing cells
observed in response to LIF was unanticipated. LIF was
initially characterized, purified, and cloned as a regulator
able to induce differentiation in the murine myeloid
leukemic line M1 (13, 51, 52) and has since been shown to
have comparable actions on the human myeloid leukemic
lines HL60 and U937 (53, 54). Although having the
opposite action of preventing differentiation in multi-
topotential embryonic stem cells (55, 56), its differentia-
tion-inducing ability is also evident in its capacity to
switch signaling of autonomic nerves from the adrenergic
to the cholinergic mode (57). The present results have
demonstrated a new target for its differentiation-inducing
action, namely certain myc-transformed erythroid cell
lines.

In the case of the myeloid leukemias, the ability of LIF
to enhance differentiation commitment in the leukemic
cells has led to consideration of the possible use of LIF
in the clinical management of these diseases. Such consi-
derations seem now to be worthy of extension to the
clinical management of the erythroleukemias.

Materials and Methods

**Viruses.** The c-myc retroviral plasmids were constructed
by insertion of the 1.4-kb XhoI fragment of murine myc
cDNA (58) into the XhoI cloning site of the retroviral
vectors pZen and pMPZen (23). Fibroblast lines secreting
c-myc virus free of helper virus were produced by trans-
fecitng the ψ2 packaging line with Sall-linearized retro-
viral plasmid DNA and pSV2Neo DNA at a molar ratio of
10:1 as previously described (48). G418-resistant clones
were screened for virus production by cocultivating 10^6
fibroblasts and 10^6 FDC-P1 cells for 2 days in DMEM
containing 10% fetal calf serum and 1% WEHI-3B condi-
tioned medium as a source of IL-3 (48). Xbal-digested
DNA prepared from the FDC-P1 cells was then analyzed
by Southern blot analysis for the presence of the ex-
pected 3.4-kb provirus. Clones R15 and M40 produced
the highest titer of Zen[myc] and MPZen[myc] virus,
respectively, as judged by the intensity of the myc pro-
vidal band compared to the endogenous myc band in the
unselected FDC-P1 population. They were routinely
propagated in DMEM containing 10% calf serum and 400
µg/ml G418 (Geneticin; Gibco).

**Infection of Fetal Liver Cells.** Liver cells (7.5 × 10^6)
from 13-day fetal CBA mice were infected by cocultiva-
tion with 10^6 irradiated (35 Gy) virus-producing ψ2 cells
plated 24 h previously in a 60-mm dish containing 5 ml
DMEM supplemented with 20% fetal calf serum, 5 × 10^{-3} M
2-mercaptoethanol, and 1% WEHI-3B conditioned medium.
Control cultures were cultivated with the parental ψ2 cells.
After 48 h, nonadherent cells from the fibroblast monolayer
were replated in a new dish for 2 h to allow attachment of any
contaminating adherent cells. They were then harvested by
centrifugation, resuspended in 10 ml fresh IL-3-containing
medium, and cultured for a further 3 days, after which
one-half of the cells were replated in medium lacking IL-3.
Most cells died over the next few days, but then, ir-
respective of whether or not IL-3 was present, the highly
characteristic erythroid cells became apparent in myc
virus-infected but not control cultures. The cells were main-
tained at relatively high density (1–7.5 × 10^5/ml) and
expanded for DNA and RNA preparation.

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**Table 1.** Transplantation of c-myc virus-infected erythroid lines

<table>
<thead>
<tr>
<th>Line</th>
<th>Days in culture</th>
<th>Recipient</th>
<th>Irradiation</th>
<th>Tumor frequency</th>
<th>Age killed</th>
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<tbody>
<tr>
<td>ME1</td>
<td>21</td>
<td>nu</td>
<td>–</td>
<td>0/2</td>
<td>198</td>
</tr>
<tr>
<td>ME2</td>
<td>15</td>
<td>nu</td>
<td>–</td>
<td>0/2</td>
<td>206</td>
</tr>
<tr>
<td>ME2.11</td>
<td>39</td>
<td>nu</td>
<td>–</td>
<td>0/2</td>
<td>182</td>
</tr>
<tr>
<td>ME3</td>
<td>28</td>
<td>nu</td>
<td>–</td>
<td>0/2</td>
<td>180</td>
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<td>ME4</td>
<td>22</td>
<td>nu</td>
<td>–</td>
<td>0/2</td>
<td>182</td>
</tr>
<tr>
<td>ME5</td>
<td>21</td>
<td>nu</td>
<td>–</td>
<td>0/4</td>
<td>134</td>
</tr>
<tr>
<td>ME11</td>
<td>21</td>
<td>nu</td>
<td>–</td>
<td>0/2</td>
<td>188</td>
</tr>
<tr>
<td>ME12</td>
<td>13</td>
<td>nu</td>
<td>–</td>
<td>0/4</td>
<td>134</td>
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<tr>
<td>ME14.C4</td>
<td>100</td>
<td>CBA</td>
<td>+</td>
<td>3/3</td>
<td>335, 382, 450</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>3/3</td>
<td>335, 528, 528</td>
</tr>
<tr>
<td>ME15.C2</td>
<td>85</td>
<td>CBA</td>
<td>–</td>
<td>3/3</td>
<td>125, 114, 129</td>
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<tr>
<td>ME17.2B</td>
<td>93</td>
<td>CBA</td>
<td>+</td>
<td>3/3</td>
<td>91, 201, 401</td>
</tr>
</tbody>
</table>

* Mice received injections of a total of 3 × 10^6 cells delivered s.c., i.p.,
and s.c.
| Ratio of tumor-bearing mice to number given injections.
| Mice killed healthy or with tumors.
| Mice transplanted in three unirradiated mice, tumors developed with a latency of 82, 126, and 135 days.
| Mice transplanted in four irradiated mice, tumors developed with a latency of 35, 34, and 35 days.
| Mice transplanted in unirradiated mice, a tumor developed in one recipient after 142 days; the other recipient died of other (nontumor) causes at 300 days.

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**Fig. 6.** Differentiation of c-myc-transformed erythroid cells. Colonies containing hemoglobinized benzidine-positive (δ+) cells after varying periods of culture in the presence of normal saline (NS) or the indicated growth factors were quantitated after staining with benzidine. ME15.C2 cultures contained 1% DMSO.
Clonal Cell Cultures. Cloning studies were performed in 35-mm plates containing 1 ml of 0.9% methylcellulose or 0.3% agar in Iscove's modified Dulbecco's medium containing 20% fetal calf serum and 5 × 10⁻⁵ M 2-mercaptoethanol as described elsewhere (59). The cultures were scored at intervals after incubation at 37°C in a fully humidified atmosphere of 5% CO₂ in air, using a dissection microscope at 35X magnification.

Growth Factors. Purified recombinant growth factors used in cultures were murine IL-3 (200 units/ml), murine GM-CSF (500 units/ml), human GM-CSF (500 units/ml, kindly provided by Amgen, Thousand Oaks, CA), murine M-CSF (1000 units/ml), human IL-6 (1000 units/ml), human Epo (2 units/ml; Amgen), and human LIF (1000 units/ml). All were derived via Escherichia coli expression vectors, except for M-CSF, which was expressed in yeast, and all had specific activities approximating 10⁶ units/mg. For factors with bone marrow colony-stimulating activity, 50 units/ml is defined as the concentration stimulating half-maximal numbers of colonies in conventional agar cultures, whereas LIF activity was defined as described elsewhere (20).

Benzidine Staining. Cytocentrifuged preparations were fixed in methanol, stained with diaminobenzidine (Sigma) and counterstained in 3% Giemsa (59). For colonies growing in agar, hemoglobinization was assessed by in situ staining of the entire agar culture with benzidine dihydrochloride (By-products and Chemicals Co., Australia) according to Cooper et al. (60). Benzidine-positive cells (deep blue) were scored 5–10 min after staining. Colonies with even a single benzidine-positive cell were scored as positive.

Filter Hybridization. DNA fragments labeled by random priming and used as hybridization probes were: c-myc, 1.4-kb XhoI fragment of pmc54 (58); β-globin, 1-kb PstI fragment of β-globin (see Ref. 37); GF-1, XhoI fragment from clone 127 (27); Epo receptor, residues 70-1556 (26), cloned by PCR by K. Klingler; spi, 1, 1250-base pair insert cDNA clone b1 (33); kit, 1560-base pair HindIII-DraI fragment of murine c-kit CDNA (28), cloned by PCR by T. Gonda.

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References

172
c-myc-transformed Erythroid Cells


