Erythroid colonies from five patients with an early erythroblastic leukemia were obtained in "serum-free" cultures in the presence or absence of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and homogeneous native erythropoietin (Epo). Erythroid colonies with abnormal morphology and karyotype could be grown in different culture conditions. Their erythroid nature was ascertained by the presence of carbonic anhydrase I and glycophorin A. Leukemic erythroid progenitors strongly differed from normal progenitors in that spontaneous colonies were always obtained, sometimes with an extremely high plating efficiency (up to 5.7%). Colonies were found to be autonomous from exogenous hematopoietic growth factors because they were still obtained with a high plating efficiency at an average of one cell per culture in the absence of any added growth factor. No evidence for an autocrine secretion of Epo or GM-CSF emerged because Epo or GM-CSF could not be detected by biologic or radioimmunologic assays from the culture supernatant or cellular extracts of the leukemic cells and that Epo or GM-CSF antibodies did not block autonomous growth.

In all cases, however, hematopoietic growth factors increased the plating efficiency of the abnormal erythroid progenitors. In the two "de novo" leukemias, leukemic erythroid progenitors responded primarily to Epo, whereas in the three other patients' (chronic myeloid leukemia) blast crisis they responded maximally to GM-CSF plus Epo. Recombinant erythroid-potentiating activity had no effect in any of these cases. These results suggest that the leukemic erythroid clonogenic cells arise from expansion of erythroid progenitors at different levels of differentiation (ie, CFU-E or BFU-E, depending upon the disease) and that autonomous growth is not related to a secretion of Epo or GM-CSF.

© 1987 by Grune & Stratton, Inc.

MATERIAL AND METHODS

Patients. The first four patients (A, B, C, and D; Table I) have been described in two previous publications and corresponded to patients no. 5, 6, 7, and 9, respectively, in these manuscripts. The fifth patient (E) is a newly diagnosed patient. Briefly, diagnosis of these five cases as erythroid leukemia was based upon the reactivity of the blast cells with an anti-CAI rabbit polyclonal antibody and with the FA6 152 MoAb.

Patients A, B, and C were chronic myeloid leukemia (CML) patients in terminal blast crisis, whereas patients D and E had apparently primitive leukemias. In patient C, the erythroid blasts were mixed in an equal proportion with blasts of the megakaryocytic series.

Samples. Peripheral blood from the erythroleukemic patients was collected into sterile syringes containing heparin. Light-density cells were separated over a Ficoll-metrizoate gradient density centrifugation (Lymphoprep, Nyegaard, Oslo) and then washed three

From Institute National de la Sante et de la Recherche Medicale (INSERM) U.91, Hôpital Henri Mondor, Creteil, France; Hôpital de la Santa Creu i Sant Pau, Barcelona, Spain; INSERM U.301, Hôpital Saint-Louis, Paris; Department of Medicine, University of California at Los Angeles; and Terry Fox Laboratory, British Columbia Cancer Research Centre, Vancouver, BC, Canada.

Submitted February 2, 1987; accepted May 26, 1987.

Supported by Groupe des Entreprises Francaises dans la Lutte contre le Cancer, Ligue contre le Cancer, and "Fondations contre la leucémie" funds. Dr Mitjavila is supported by Spanish and Catalan funds (Fondo de Investigaciones de la Seguridad Social and Comissió Interdepartamental de Recerca i innovació Tecnològica).

Address reprint requests to W. Vainchenker, MD, INSERM U.91 Hôpital Henri Mondor, 94010 Creteil, France.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1987 by Grune & Stratton, Inc.

0006-4971/87/7004-0016$03.00/0

Blood, Vol 70, No 4 (October), 1987: pp 965-973
times in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Paisley, Scotland). The cells subsequently were cryopreserved in pure fetal calf serum with 10% dimethylsulfoxide (Lab Bruneau, Paris) by using either a minicooler (Compagnie Francaise de Produits Oxygènés, Paris) or a standard technique (–20°C for two hours, –70°C overnight) and kept in liquid nitrogen until use.

Marrow cells were obtained from normal marrow donors after informed consent, and an 18-day-old fetal liver was studied after separation of the light-density cells on a Ficoll-metrizoate gradient as before.

**Semisolid culture procedures.** Cultures were maintained in "serum-free" medium by using the techniques described by Iscove et al.17 and Steward et al.18 as applied to human cells. Briefly, the medium contained 1.5% denized bovine serum albumin (Cohn fraction V, Sigma Chemical Co, St Louis),19 iron-saturated human transferrin (300 μg/mL, Sigma), calcium chloride (28 μg/mL), a mixture of sonicated lipids, 7.5 × 10⁻⁴ mol/L α-thioglycerol, 100 ng/mL insulin, and 0.8% 4,000 cp methylcellulose in IMDM. The mixture of lipids was obtained by sonicating 7.8 mg cholesterol, 6.14 mg linoleic acid, and 7.4 mg dipalmityl lecithin (all obtained from Sigma) in 10 mL of IMDM (without sodium bicarbonate) containing 1% serum albumin as previously described.17 Twenty microliters of this mixture was used per milliliter culture. Control cultures were maintained with 10% human AB serum or fetal calf serum. Some cultures were cytocentrifuged and fixed with pure methanol for one minute.

**Immunofluorescence studies on cells grown in culture.** Cultures grown in methylcellulose with different stimulating factors were individually recovered by adding 1 mL of Hanks' medium to each Petri dish. The cells were washed and counted and their viability evaluated by the trypan blue exclusion test. Subsequently, the cells were cytacentrifuged and fixed with pure methanol for one minute. Double immunofluorescence labeling was performed by first using a pool of three murine anti-glycophorin A (GPA) MoAbs (LICR-LON-R102, LICR-LON-R183 and H85 [Immunotech, Marseille, France]) at a 1/100 dilution followed by a goat F(ab) fragment against mouse Ig conjugated to fluorescein (Bioart, Meudon, France); then the rabbit polyclonal antibody against CAI was applied and revealed by goat IgG against rabbit IgG conjugated to rhodamine. Slides were mounted and observed under a Zeiss microscope equipped with appropriate filters.

The absolute number of CAI-positive cells was calculated by multiplying the total number of cells per culture by the percentage of CAI-positive cells after fluorescence labeling. In all cases, cytocentrifuge preparations were stained with May-Grünwald-Giemsa stain.

When cultures were performed at limiting dilution, some colonies were individually pipetted out of the microwells and transferred to a 0.2-mg/mL polylsine (Sigma)-coated slide, and then fluorescence labeling was performed with the anti-GPA MoAbs. Plasma clot cultures were also labeled in situ by the same antibodies as previously reported.12

**Karyotypic analysis of the erythroid colonies.** Patients A, B, and D were previously cytogenetically characterized. Patient C has only a Philadelphia (Ph₁) chromosome. Karyotypic analysis of colonies was performed to distinguish clearly the abnormal erythroid clones from the others. The studies were performed with cells from patients A, B, and D. Metaphases from individual colonies were analyzed essentially as described by Dubé et al. Briefly, each culture was monitored for the optimal selection time (ie, day 5 for patient A and day 9 for patients B and D). One hour before harvesting, 0.1 mL of colcemid (GIBCO) diluted to 1 μg/mL was distributed over the culture. Individual colonies were harvested and directly transferred to polylsine-coated slides covered with 2 drops of 0.075 mol/L KCl. After 20 minutes in a humid environment at

### Table 1. Characteristics of the Five Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Primary Syndrome</th>
<th>Age</th>
<th>Sex</th>
<th>Leukocytes</th>
<th>Blasts (%)</th>
<th>Erythroid Blasts After Ficol Separation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CML</td>
<td>34</td>
<td>M</td>
<td>103,000</td>
<td>63</td>
<td>90</td>
</tr>
<tr>
<td>B</td>
<td>CML</td>
<td>58</td>
<td>F</td>
<td>82,000</td>
<td>19</td>
<td>40-60†</td>
</tr>
<tr>
<td>C</td>
<td>CML</td>
<td>17</td>
<td>M</td>
<td>52,000</td>
<td>23</td>
<td>35†</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>58</td>
<td>M</td>
<td>18,000</td>
<td>47</td>
<td>80</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>62</td>
<td>M</td>
<td></td>
<td>45</td>
<td>75</td>
</tr>
</tbody>
</table>

*Marrow specimen.
†These percentages were raised after thawing of frozen samples. A variation was observed according to the sample for each patient.

In addition, leukemic cells from patients C and D were incorporated at 1 × 10⁶ cells/mL in a 0.5% agar overlay. A methylcellulose overlay containing 5 × 10⁶ normal marrow cells was subsequently added, and colonies were observed at days 7 and 12.

In some experiments, rabbit anti-GM-CSF serum or rabbit anti-Epo serum was directly included in the culture. The GM-CSF and Epo antibodies were obtained by immunizing rabbits with recombinant GM-CSF or homogenous native Epo. A quantity of 5 μL/mL of anti-Epo serum was able to neutralize the activity of 10 IU of exogenous Epo; the anti-GM-CSF serum was used at a 1/100 dilution. Preimmune serum was used as a control. Because cultures from patient D were inhibited by the serum, the anti-Epo IgG fraction was separated from anti-Epo serum by diethylaminoethyl–Aib-Blue gel chromatography (Bio-Rad Laboratories, Richmond, Calif.).
Fig 1. Aspect of abnormal erythroid colonies from the five patients. Cells were grown in serum-free methylcellulose cultures, and colonies were observed under an inverted microscope except for patient A. (A) A colony observed in patient A at day 12 after stimulation by GM-CSF (original magnification ×1,400; current magnification ×1,400). (B) A spontaneous colony observed in patient B at day 12 of culture (original magnification ×1,400; current magnification ×1,400). (C) A similar colony grown in patient B in plasma clot culture and in situ labeled with the polyclonal antibody against CAI after methanol fixation of the culture. Binding was revealed by goat IgG against rabbit IgG conjugated to rhodamine and observed under an episcopic at low magnification (original magnification ×700; current magnification ×700). (D) A large erythroid colony grown from patient C in the presence of 100 pmol/L GM-CSF and 1 IU/mL Epo (original magnification ×400; current magnification ×400). (E) A spontaneous colony from patient D (original magnification ×1,400; current magnification ×1,400). (F) An erythroid colony (in patient D) normally hemoglobinized (appearing dark on the photograph) is in close proximity to an abnormal erythroid colony in a culture stimulated by Epo (original magnification ×1,400; current magnification ×1,400). (G) A small colony (in patient E) grown in the presence of Epo (original magnification ×2,800; current magnification ×2,800).

In patient A, the colonies were medium sized (50 to 200 cells) and relatively dispersed and maximum at day 12. In patient B, the colonies were large, dispersed, and maximum between days 12 and 18. At this time, a portion of the colonies became much tighter, especially when Epo was added, and they resembled typical BFU-E colonies. In patient E, the colonies were small (up to 50 cells), compact, composed of large cells, and maximum at day 8. The erythroid origin of the colonies from these three patients was established by labeling these cells with erythroid differentiation markers both in plasma clot, which had the advantage of labeling all colonies in situ, and in methylcellulose cultures. Cells from these colonies exhibited CAI; in addition 40% were labeled by anti-GPA MoAbs. Epo significantly increased the proportion of GPA-positive cells.

RESULTS

Characterization of the erythroid colonies. Preliminary results have shown that abnormal erythroid colony formation could be obtained in these five patients under different culture conditions (Fig 1). However, these colonies were atypical in patients A, B, and E, whatever the culture conditions.

room temperature, the excess hypotonic solution was gently removed, and the slides were fixed with a mixture of methanol-acetic acid (3:1). The slides were then allowed to dry and were Giemsa stained (pH 6.8) to identify the mitoses. R-bands, using giemsa after heating, (RHG) were performed without destaining. All mitoses were examined by using a phase-contrast microscope and photographed. One to seven metaphases per colony were studied.
In patients C and D, the erythroid colonies were much more typical. A spectrum of erythroid colonies corresponding to early and late BFU-E colonies was present in patient C. In patient D, all the erythroid colonies were similar and looked like late BFU-E. Although more typical in appearance, these colonies were abnormal because they were never as red as the rare colonies with a normal karyotype (Fig 1). In these two patients, cells exhibited both GPA and CAI even in the absence of Epo. This factor markedly increased the terminal maturation of erythroblasts in patient C, as attested by morphological studies.

In patients D and E, only erythroid colonies were observed under a variety of culture conditions. In patients A, B, and C, on the other hand, granulocytic (neutrophilic, basophilic, and eosinophilic) colonies also grew; additionally, a large number of megakaryocytic colonies were seen in patient C even in the absence of a specific stimulating factor. The granulocytic colonies of patients A, B, and C were observed at low cellular concentration only when GM-CSF was added. Mixed erythrogranulocytic colonies were not observed.

Cytogenetic analysis of the erythroid colonies. Cytogenetic studies of bone marrow (patient A) or of unstimulated blood cultures (patients B and D) have been reported previously. All metaphases from patient A were hyperdiploid (56 to 64 chromosomes) including a standard Ph, chromosome. All metaphases from patient B were abnormal: some had the Ph, as the sole abnormality; others had previously. All metaphases from patient A were hyperdiploid (56 to 64 chromosomes) including a standard Ph, chromosome. All metaphases from patient B were abnormal: some had the Ph, as the sole abnormality; others had previously. All metaphases from patient A were hyperdiploid (56 to 64 chromosomes) including a standard Ph, chromosome. All metaphases from patient B were abnormal: some had the Ph, as the sole abnormality; others had previously. All metaphases from patient A were hyperdiploid (56 to 64 chromosomes) including a standard Ph, chromosome. All metaphases from patient B were abnormal: some had the Ph, as the sole abnormality; others had previously. All metaphases from patient A were hyperdiploid (56 to 64 chromosomes) including a standard Ph, chromosome. All metaphases from patient B were abnormal: some had the Ph, as the sole abnormality; others had previously.

Results of the cytogenetic study of colonies are summarized in Table 2. Cells from spontaneous colonies (patients B and D) and from colonies grown in the sole presence of GM-CSF (patient A) showed either hyperdiploid Ph, positive karyotypes (except in one colony from patient B) or abnormal karyotypes with the marker chromosome (patient D). The colonies grown in the presence of Epo were hyperdiploid in patient A, either diploid and Ph, positive or hyperdiploid and Ph, positive in patient B, and either abnormal (with the marker chromosome) or normal in patient D.

Dispersed and tight colonies were not cytogenetically distinguishable in patient B.

These results showed that some colonies resulted from blastic cells (hyperdiploid in patients A and B, with a marker in patient D), whereas the others resulted from Ph, positive CML chronic-phase cells (granulocytic or erythroid) in patient A and B or from apparently normal cells in patient D (normal karyotype and colonies with a normal appearance).

From this study, it appeared that all colonies from patient A, whatever the culture conditions, belonged to the acute phase of leukemia; in patients B and D, erythroid progenitors from the CML chronic phase or from normal clones were estimated to be less than 5% because these karyotypes were only found in erythroid colonies with a normal aspect.

Table 2. Cytogenetic Studies of Colonies

<table>
<thead>
<tr>
<th>Patients</th>
<th>Stimulating Factors</th>
<th>Types of Picked Colonies</th>
<th>Number of Examined Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>GM-CSF</td>
<td>Dispersed</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Epo</td>
<td>Dispersed</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>Epo</td>
<td>Dispersed</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Epo</td>
<td>Tight</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>Epo</td>
<td>Single</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Epo</td>
<td>Pooled</td>
<td>(18)*</td>
</tr>
</tbody>
</table>

Cells were plated in 1 mL Petri dishes in serum-free conditions in the presence or absence of stimulating factor. Colonies were picked as single colonies under an inverted microscope, or colonies were pooled. The karyotype was then studied.

*Numbers in brackets are the numbers of metaphases analyzed.
REGULATION OF LEUKEMIC ERYTHROID GROWTH

Fig 2. Dose-response curve to recombinant GM-CSF and EPA that illustrates the growth of leukemic erythroid colonies from patient C in the absence of Epo. Cells were cultured in serum-free conditions at 15 x 10⁴ cells/mL in triplicate, and colonies were enumerated under an inverted microscope at day 12. Because the maturation of the cells was more complete than in the other patients, colonies were easier to enumerate. It is noteworthy that in this experiment the number of spontaneous colonies was quite low. Cells were subsequently recovered, counted, and cytoospinned. Slides were stained by the May-Grünwald staining, and mature and immature erythroblasts were enumerated. The total number of erythroid cells was obtained by multiplying the total number of cells by the frequency of erythroblasts (~80%).

In these patients, neither EPA alone nor EPA combined with Epo and/or GM-CSF had a clear effect on either the number of colonies or on cellular proliferation as expressed in the number of erythroid cells (CAI-positive cells) per culture (Figs 4 and 5). However, in all cases except patient A, Epo clearly enhanced both the plating efficiency and the size of the colonies in comparison to spontaneous colonies. In patient A, Epo significantly increased the number of colonies in comparison to unstimulated cultures in only two of eight experiments. In the remaining six experiments, the number of colonies was slightly but not significantly higher than in the absence of Epo. The results of a typical experiment are represented in Figs 4 and 5. In patients B, D, and E, colonies with a normal appearance were also observed after addition of Epo, but were rare (less than 1% of the morphologically abnormal colonies).

Fig 3. Dose-response curve illustrating the effect of homogeneous Epo in the presence of 100 pmol/L recombinant GM-CSF on the growth of leukemic erythroid colonies from patient C. The experiment was identical to that reported in Fig 2 except that the cells were plated at 1 x 10⁵ cells/mL. The frequency of erythroid cells was more than 85% for 1 IU/mL of Epo.
that of normal erythroid colonies. However, they were never frankly red. In patient C, Epo was also added to GM-CSF-stimulated cultures at day 4 of culture, and the plating efficiency was reduced approximately 30% in comparison to GM-CSF-stimulated cultures containing Epo at the onset of the cultures (Fig 6). This loss in the number of colonies was identical to the number of colonies obtained with Epo alone (Fig 6), and the remaining colonies corresponded to the biggest colonies under the optimal conditions.

Therefore, leukemic erythroid progenitors from patients D and E only responded to Epo, whereas those of patients A, B, and C responded maximally to the combination of GM-CSF and Epo. This result was confirmed by studying the relationship between the number of plated cells and the number of colonies. In patients D and E, a linear curve extrapolating to zero defined the relationship between the number of colonies and the number of plated cells when Epo was added alone. A similar curve was found in patient C only when cells were cultured in the presence of GM-CSF plus Epo (data not shown).

The effect of normal human serum and fetal calf serum was tested in all patients. In patients B and E, serum had no significant effect, but in patient C it clearly potentiated the effect of Epo. Surprisingly, the addition of serum markedly inhibited erythroid colony formation in patients A and D. In patient D, serum (both human and fetal calf) at a concentration as low as 40 μL/mL completely inhibited abnormal erythroid colony formation.

GM-CSF alone had a very significant effect in all patients (increasing up to ten times the number of colonies) except patients D and E. The colonies had the same appearance as spontaneous colonies but were larger.

In patients B and C, Epo and GM-CSF acted additively, increasing the number and the size of these colonies, and their combined action gave them an appearance similar to
Finally, cells from patients were grown at low dilution (average of one cell per well) in individual wells, which resulted in the presence of one cell per well (Table 3). Spontaneous colonies were obtained with a plating efficiency of 1% in patients C and D and 5.7% in patient B. Results paralleled those previously observed in standard cultures. Specifically, progenitors from patient D only responded to Epo, whereas those from patients B and C maximally responded to the combination of Epo and GM-CSF.

Because autonomous growth was observed, we tried to determine whether leukemic cells were secreting a CSF or Epo.

Conditioned medium by leukemic cells or crude cellular extracts were screened for such activities. In none of the patients was a CSF or Epo activity found when using normal human marrow cells as the target. In addition, when leukemic cells from patients C and D were used in an underlayer, they did not promote the growth of CFU-E. Radioimmunoassays for GM-CSF and Epo (kindly performed by Dr. E. Goldwasser, University of Chicago) were negative on all conditioned media and cellular extracts.

Anti-Epo serum from 2 to 10 μL/mL was added to the cultures from patients B and C and did not prevent the growth of spontaneous erythroid colonies, whereas it blocked the action of exogenous Epo. In patient D, 5 μL of rabbit serum (immune or preimmune) completely inhibited colony growth. Therefore, rabbit anti-Epo IgG was added to the culture and did not block the spontaneous growth. Moreover, in these three patients, the addition of Epo antibodies did not prevent acquisition of GPA or of Hb by leukemic cells in culture. GM-CSF antiserum was added in culture from patients A and B and did not block spontaneous growth.

**DISCUSSION**

Using anti-GPA antibodies and earlier markers of erythroid differentiation, we had the opportunity to diagnose ten cases of early erythroid leukemia during an 18-month period. In eight of these, in vitro studies could be performed. In three patients with Down's syndrome, no colony growth could be obtained with any culture procedures (data not shown). In the five patients reported here, leukemic erythroid colony-forming cells could be grown. The leukemic erythroid progenitor cells from the three patients in blast crisis of CML maximally responded to the association of Epo and GM-CSF. Because autonomous growth was observed, we tried to determine whether leukemic cells were secreting a CSF or Epo.

Table 3. Plating Efficiency of Leukemic Erythroid Progenitors at an Average of One Cell per Well

<table>
<thead>
<tr>
<th>Patient</th>
<th>Epo (IU/mL)</th>
<th>GM-CSF (100 pmol/L)</th>
<th>GM-CSF + Epo (100 pmol/L + 1 IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>5.7</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>6</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Cells were diluted to 10 cells/mL in serum-free cultures and plated in a 100 μL volume. Six hundred microwells for patient B and 300 microwells for patients C and D were plated without exogenous stimulating factor, whereas 200 microwells were plated in all the other culture conditions.

amplified this action and favored terminal differentiation. Therefore, in these three cases, a large part of the leukemic erythroid clonogenic cells behaves as a normal primitive BFU-E. In addition, we have presented evidence that a spectrum of leukemic erythroid clonogenic cells was present in two of these patients. Indeed, large and small abnormal colonies bearing the same cytogenetic marker could be grown with GM-CSF plus Epo and with Epo alone, respectively.

In the two last patients with de novo leukemias, the erythroid leukemic clonogenic cells only require Epo to form colonies and differentiate. GM-CSF or any conditioned media tested (data not shown) (Mo medium, phytohemagglutinin–leukocyte-conditioned medium, or supernatant from the 5637 cell line) were unable to stimulate growth of these cells, which therefore behave as normal CFU-E or a late BFU-E.

In none of these patients did EDA elicit a response. The action of EDA as a growth factor is actually a matter of debate because it has a homologous structure with a metalloproteinase inhibitor and controversial results have been obtained on the growth of normal erythroid progenitors. We are currently investigating the action of RNA on normal cells to explain the absence of response to EDA of leukemic erythroid cells.

The present results are consistent with those previously obtained in acute myeloblastic leukemia (AML). Indeed, a marked heterogeneity in the AML clonogenic cell among different patients was attested to by the surface antigen phenotypes of these cells. In addition, in the same patient an AML cluster–forming cell and a colony-forming cell are present, which suggests the presence of a spectrum of leukemic progenitors. The AML clonogenic cells also respond to recombinant GM-CSF; however, it is not yet known whether their heterogeneity reflects distinctive CSF requirements.

In this study, we could demonstrate that leukemic erythroid progenitors differ from their normal counterparts by a relative independence to growth factors, especially to Epo. In all these patients, more or less spontaneous colonies could be grown that appeared to be really autonomous. Indeed, they were obtained in a serum-free medium that, however, contained albumin and still grew at a very low cellular density, excluding the possibility that their growth was regulated by an accessory cell. Interestingly, karyotypic analysis in a CML patient has shown that in addition to the Ph1 chromosome all spontaneous colonies except one bear the cytogenetic markers of the blast crisis. This result supports the contention that autonomous growth has been acquired during clonal evolution toward the erythroid blast crisis and was not a growth characteristic of the initial chronic phase.

Spontaneous erythroid colonies have already been described in some human erythroleukemias (M6 of the French-American-British classification and related disorders) and some myeloproliferative diseases (e.g. in polycythemia vera in which it is a sensitive criterion for diagnosis). In this latter disease, it was previously suggested that the spontaneous colonies were hypersensitive to Epo. However, a recent study affords strong evidence for their true Epo independency.
In animal models, three different mechanisms have been proposed to explain growth factor–independent proliferation. The first is autocrine secretion of the growth factor; the second is modification of the intracellular transmission pathway of the growth factors; and the third is modification of a growth factor receptor. Previous studies in human AML have shown that leukemic and normal cells have similar growth requirements. This finding led to the suggestion that AML might be a growth factor–dependent rather than an autonomous neoplasm. Recently, Young and Griffin reported three cases of autonomous growth among 15 AML patients. Two of them were related to an autocrine secretion of GM-CSF. The experiments reported here did not afford any evidence for autocrine secretion of a hematopoietic growth factor because neither CSF nor Epo activity was detected in conditioned media from leukemic cells. In addition, GM-CSF or Epo antibodies did not block the growth of spontaneous colonies or even the terminal differentiation of leukemic cells. Therefore, these data suggest that one of the two other previously mentioned mechanisms may bypass the growth factor requirement. However, very recently in a few cases of AML, Young et al. have reported the presence of GM-CSF transcripts in the absence of detectable active CSF in conditioned media from leukemic cells. Future investigations will have to exclude the possibility that the Epo or other hematopoietic growth factor genes are constitutively expressed in some erythroleukemia without detection of the growth factor itself.

Further studies will be required to determine which mechanism is operating in human erythroleukemia.

Autonomous growth was not the only difference from normal cells. The human erythroleukemic cells also differ by a relative blockage in their terminal differentiation. This blockage is more or less important, depending upon the particular patient, but exogenous Epo facilitated terminal differentiation. Therefore, in erythroleukemia Epo had a differentiation action similar to G-CSF in AML. In contrast, GM-CSF did not induce more complete differentiation than the spontaneous one.

Finally, it has to be pointed out that in two cases normal serum from several species could inhibit or abolish the leukemic erythroid growth, which suggests the presence of a powerful inhibitor of this growth in serum. Experiments currently performed in patient D suggest that Transforming Growth Factor-β (TGF-β) is involved in this negative regulation (manuscript in preparation). Serum inhibitor of cell proliferation may also explain the absence of growth of some AML.

In conclusion, in five cases of erythroleukemia we showed that the leukemic erythroid clonogenic cells were the equivalent of CFU-E in two cases of de novo leukemia and BFU-E in three cases of CML blast crisis. These cells differed from normal cells by both an autonomous growth and a relative blockage in their terminal differentiation. The autonomous growth could not be explained by an autocrine secretion of Epo or another hematopoietic growth factor. From this study, it appears that human erythroleukemia can be compared with the avian model. In the chicken, two viral oncogenes act synergistically to cause erythroleukemia. The first one, v-erb B, gives the proliferative advantage, whereas the second one, v-erb A, blocks the erythroid cell terminal differentiation, thereby giving the phenotype of a CFU-E.

ACKNOWLEDGMENT

We are grateful to Dr A.E.W. Edwards (London) for providing LICR-LON-R10 and LICR-LON-R18, Dr Mannoni (Marseille, France) for H85, and Dr E. Goldwasser (Chicago) for performing the Epo-radio immunoassays.

We thank A.M. Dulac for preparation of the manuscript and the figures, J.M. Masse for photographic assistance, and J. Lewis for improving the manuscript.

REFERENCES

17. Iscove NN, Guilbert Li, Weyman C: Complete replacement of serum in primary cultures of erythropoietin-dependent red blood cell precursors (CFU-E) by albumin, transferrin, iron, unsaturated fatty acid, lecithin and cholesterol. Exp Cell Res 126:121, 1980
Effects of granulocyte-macrophage colony-stimulating factor and erythropoietin on leukemic erythroid colony formation in human early erythroblastic leukemias

MT Mitjavila, JL Villeval, P Cramer, A Henri, J Gasson, G Krystal, M Tulliez, R Berger, J Breton-Gorius and W Vainchenker

Updated information and services can be found at:
http://www.bloodjournal.org/content/70/4/965.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml