Factor-Dependent Erythroid Cell Lines Derived From Mice Transplanted With Hematopoietic Cells Expressing the v-src Oncogene

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Transplantation of spleen cells from primary reconstituted mice expressing the v-src oncogene to secondary and tertiary irradiated recipients resulted in the emergence of erythroid precursors with a transformed phenotype. When cultured in methyl cellulose, these precursors generated colonies of undifferentiated cells that could be expanded into continuously growing factor-dependent cell lines in liquid culture. All lines tested had a similar phenotype and expressed the v-src oncogene. In addition they responded to factors that regulate normal erythroid development, namely erythropoietin (Epo), interleukin-3 (IL-3), and mast cell growth factor (MGF), the ligand to the c-kit encoded receptor. When cells from one of the lines were maintained in the absence of factor, a “factor independent” subpopulation emerged that appeared to grow in an autocrine fashion. Conditioned medium from these cells stimulated their own growth as well as the growth of broad spectrum of normal precursors. Studies with neutralizing antibodies indicated that the predominant colony-stimulating factor produced by these cells is IL-3.

The hematopoietic system is maintained throughout life by a population of primitive stem cells that have the capacity to generate differentiated progeny within multiple blood cell lineages as well as the potential to generate more stem cells through a process known as self-renewal. The mechanisms involved in the development of these diverse hematopoietic lineages from a common stem cell pool are poorly understood. Proto-oncogenes are of interest in this context as they play key roles in various stages of signal transduction and growth control, and a number of them, in particular those which encode growth factor receptors, are involved in the development and growth of hematopoietic cells.

However, there is a large group of proto-oncogenes whose function within the hematopoietic system remains unknown. The c-src gene that encodes a membrane-associated protein with tyrosine kinase activity falls into this category. With the exception of platelets and developing monocytes, c-src is not highly expressed in hematopoietic cells. Nevertheless, a large number of studies have demonstrated that expression of the activated viral form of the gene, v-src, can alter the growth and development of hematopoietic cells both in vivo and in vitro, suggesting that it is involved in normal hematopoietic differentiation. In the avian system, expression of v-src in hematopoietic cells results in a partial arrest of differentiation of erythroid precursors both in culture and in animals, leading to extensive proliferation of these cells. In concert with another differentiation arresting oncogene, v-src can cause overt leukemic transformation in vivo.

In primary murine hematopoietic cells, expression of v-src does not dramatically affect the differentiation potential of the various precursor populations, but rather leads to an abnormal proliferation of these cells. Anderson and Scolnick first reported that injection of a v-src containing virus into young mice induced an erythroproliferative disease characterized by splenomegaly and severe anemia. In a subsequent study, Anderson et al found that injection of hematopoietic cells in culture with this virus led to an increased growth of erythroid precursors. Several other groups have shown that expression of v-src in long-term bone marrow cultures increased both the total cell number as well as the number of precursors within these cultures. The development of transformed cell populations was not observed in any of these studies. Transformation was demonstrated in only one study in which the v-src infected cells were cultured on a fibroblast feeder layer for several months. During this long culture period, other molecular changes could have occurred that ultimately led to the development of these transformed populations. In a previous study, we found that mice reconstituted with bone marrow cells expressing the v-src oncogene developed a severe myeloproliferative disease associated with increased extramedullary hematopoiesis. Despite this alteration of hematopoiesis, the precursor cells within these animals appeared normal with respect to their growth factor requirements and differentiation potential. This finding further supports the notion that expression of v-src in primary murine hematopoietic cells leads to their abnormal proliferation but is not sufficient to significantly alter their differentiation potential. In contrast to its effects on primary cells, expression of v-src in established factor-dependent cell lines abrogates their factor dependence and in some instances results in transformation to a tumorigenic phenotype.

To determine whether or not expression of v-src could be the first step in a series of events that ultimately leads to the transformation of hematopoietic cells in vivo, we passaged spleen cells from the primary recipients suffering from a v-src–induced myeloproliferative disease to secondary and tertiary hosts. Recipients of spleen cells from three different animals developed a leukemic-like disease characterized by the emergence of blast cell populations in the peripheral blood, spleen, and bone marrow. Precursors from these animals appeared to be blocked in their developmental pathway, as they generated colonies of undifferentiated cells in methyl cellulose cultures. Continuously grow-
ing factor-dependent cell lines were readily established from these colonies. In this report we characterize these cell lines as belonging to the erythroid lineage and demonstrate that they proliferate in response to factors that regulate normal erythropoiesis.

**MATERIALS AND METHODS**

Establishment of v-src immortalized cell lines. Spleen and bone marrow cells from secondary and tertiary recipients were cultured in methyl cellulose in the presence of interleukin-3 (IL-3) and erythropoietin (Epo). Colonies containing no obvious differentiated hematopoietic cells were picked and transferred to 24-well tissue culture plates in medium consisting of Iscove's Modified Dulbecco's Medium (IMDM), 5% fetal calf serum (FCS), IL-3 (100 U), Epo (1 U), bovine serum albumin (BSA; 1 mg/mL), and transferrin (30 μg/mL). Almost all the colonies that were transferred grew under these conditions in the liquid cultures. A number of these populations were maintained in these cultures and after a period of slow growth could be expanded into continuously growing lines. Cell lines derived from individual colonies are referred to with the prefix "E" followed by the number of the colony. Other lines were generated from pools of colonies and they are referred to as "EP" (erythroid population) followed by the number of the culture from which they were derived. Cell lines E4 and E13 were derived from mouse src-31.1* while E19, E25, E27, E31, and E45 originated from src-31.2**. (A single asterisk denotes a secondary recipient; a double asterisk a tertiary recipient.) Two populations consisting of pooled colonies (EP21 and EP28) were derived from src-27.1** whereas a third population, EP23, was derived from mouse src-25.1*.

**Antibodies.** The following antibodies were prepared as ammonium sulphate precipitates from tissue culture supernatants of the appropriate hybridomas: M1/70 which detects the Mac-1 antigen on mouse neutrophils,23 T:24 which detects the Thy-1 antigen, J11D which detects a heat stable antigen found on the majority of bone marrow cells,24 and E13 161-7 which detects the Sca-1 antigen on primitive precursors and stem cells.25 A rat monoclonal to mouse IL-3 was kindly provided by Dr. J. Abrams (DNAX, Palo Alto, CA). The anti-chicken histone H5 antibody has been described previously.26

**DNA and RNA analysis.** High molecular weight DNA was digested with the appropriate enzymes, the fragments separated in a 0.6% agarose gel and then transferred to a nylon membrane using standard techniques. Poly (A) + RNA was isolated using an oligo-(dt)-cellulose (Boehringer Mannheim GmbH, Mannheim, Germany) column, fractionated in a 1.0% agarose-formaldehyde gel and transferred to a nylon membrane. The membranes were hybridized with the appropriate [3H]-labeled cDNA probes.

**Kinase assay.** v-src-specific kinase activity was assayed essentially as described by Collett and Erikson using a polyclonal antibody against v-src (kindly provided by S. Courtneidge, Heidelberg, Germany) for immunoprecipitation.

**Growth factors.** Recombinant human Epo was purchased from Clag A.G. (Schaffhausen, Switzerland) and recombinant mouse IL-3 and IL-4 were provided as conditioned medium of the producer X63 Ag8-653 myeloma cells transfected with vectors expressing either IL-3 or IL-4.27 In the initial studies outlined in Fig 1, conditioned medium from WEHI-3B cells was used as a source of IL-3. For all other studies the above mentioned source of recombinant IL-3 was used. Mouse G-CSF, M-CSF, and GM-CSF were kindly provided by Dr N. Gough (Melbourne, Australia) and IL-1 was obtained from Dr P. Lomedico (Roche, Nutley, NJ). Mast cell growth factor (MGF) was a gift from Dr David Cosman (Immunex, Seattle, WA). Recombinant mouse IL-6 was provided by Dr L. Aardern (Amsterdam, The Netherlands). Activities for these factors have been determined as follows: MGF, IL-3, G, M, and GM-CSF by their ability to stimulate the growth of normal precursors in methyl cellulose cultures; IL-4 by its ability to

![Fig 1. Epo and IL-3 dependence for proliferation of src erythroblast lines. (A) Cells from the src erythroblast lines E4 (left) and E31 (right) were cultured in presence of Epo (10 U/mL, squares) IL-3 (20% WEHI conditioned medium, circles), or no factor (triangles). Cell numbers were determined daily in a Coulter Counter and cumulative cell numbers calculated as described in Materials and Methods. (B) Cells from lines E4 (circles) or E31 (squares) were cultured in 96-well plates with different amounts of Epo or IL-3 and processed for 3H-thymidine incorporation as described in Materials and Methods.](image-url)
synergize with IL-3 in the growth of mast cells; IL-1 by its ability to synergize with IL-3 in the stimulation of pluripotential precursors from the marrow of 5-Fluorouracil–treated mice, and IL-6 by its ability to stimulate the growth of the B-cell hybridoma B13.29D. The units for each of the factors are defined as follows: Epo, international standard units; IL-3 and IL-6, 1 U is the amount required to achieve ½ maximum stimulation of normal precursor growth or proliferation of the appropriate cell line; G, M, and GM-CSF, 50 U is the amount required to stimulate ½ the maximum number of precursors in a methyl cellulose culture; IL-1, 1,000 U is the amount required to stimulate maximum numbers of pluripotential precursors in the presence of IL-3; IL-4, a final concentration of 1% conditioned medium from X63 Ag8-653 producer cells represents saturating amounts of factor in the stimulation of mast cells in the presence of IL-3.

Methyl cellulose cultures. Cells were grown in 1-mL cultures (35-mm Petri dishes) that contained 1.0% methyl cellulose in IMDM, 4% FCS, 10 mg/mL detiolipidated, and deionized BSA, 300 μg iron-saturated transferrin plus a mixture of the following lipids: dipalmitoyl phosphatidyl-choline (22.4 μg), cholesterol (23.5 μg), and oleic acid (17.3 μg). Growth factors were added as indicated.

Liquid cultures. The erythroid cell lines were routinely maintained in IMDM with 4% FCS, 1 to 10 mg/mL detiolipidated BSA, 30 μg transferrin, and 2 to 10 U/mL human recombinant Epo at a density of at least 1 × 10⁶ cells per mL. Friend erythroleukemic cells (a kind gift of P. Charruy, EMBL, Heidelberg, Germany) were grown in the same medium, without Epo.

Differentiation induction and assay. To induce differentiation with dimethyl sulfoxide (DMSO) or butyric acid, cells were seeded at 1 × 10⁶ cells/mL in the above medium plus 1 μg/mL insulin in the presence of 2% (vol/vol) DMSO or 2 mmol/L butyric acid. The medium was changed daily. Cells were analyzed for hemoglobin production by acid benzidine staining as described earlier.34,35

Proliferation assays. To measure proliferation of the erythroid cell lines in response to Epo or IL-3, two different assays were used. First, cells were seeded at 1 × 10⁶ cells/mL in the above medium (8% FCS, 10 mg/mL BSA) and cultured in presence or absence of Epo (10 U/mL) or IL-3 (20% WEHI-3B conditioned medium). At daily intervals, cells were counted and the cultures readjusted to a concentration of 1 × 10⁶ cells/mL by addition of fresh medium. Cultures which showed little growth were subjected to daily partial medium changes and the adjustment of cell density was done by reducing culture size. Cumulative cell numbers were calculated and plotted against time.

To determine proliferation by [H]-thymidine incorporation, a variation of the chicken myelomonocytic growth factor (cMGF) assay36 was used. Briefly, 10,000 to 20,000 cells were seeded in 96-well microtiter plates into which 100 μL of the above medium containing serial dilutions of Epo and IL-3 had been dispensed. After 2 days, cells were labeled with [H]-thymidine (0.8 μCi/mL) for 2 hours and harvested in a cell harvester as described previously.37

Immunofluorescence. Staining of fixed erythroblast cell lines, control cells (avian erythroblasts, HD 3,38 murine erythroid cells, Friend erythroleukemic cells, and murine myeloid cells [WEHI-3B]) was performed as described earlier39 except that PBG (phosphate-buffered saline containing 1% detiolipidated BSA and 0.5% [wt/vol] gelatin) was used for the postfixation block as well as for the washes to reduce background staining.

RESULTS

Derivation and establishment of the v-src immortalized cell lines. Spleen cells from three different mice (src-25, src-27, and src-31) reconstituted with v-src–expressing bone marrow cells were used to reconstitute secondary irradiated recipients for various periods of time. At the time the cells were passed, all of these primary recipients had an enlarged spleen and were suffering from a myeloproliferative disease as detailed in an earlier report.17 The spleens from these secondary recipients were further passaged to tertiary recipients. In each of the series of transfers, either the secondary or tertiary transplanted animal developed a leukemic-like disease characterized by the presence of cells with a blast morphology in the spleen, peripheral blood, and bone marrow. Many of these cells had the appearance of erythroblasts. The hematopoietic tissues of these mice also contained abnormal or "transformed" precursors that appeared to be blocked in their differentiation potential as demonstrated by their capacity to generate colonies consisting of undifferentiated blast cells in methyl cellulose cultures. These colonies developed in cultures that contained IL-3 and Epo or only Epo. No colonies developed in the absence of factors, indicating that, although the precursors that gave rise to them appear to be blocked in their developmental pathway, they remained growth factor dependent. None of the control animals that were transplanted with spleen cells expressing only the neo gene showed these abnormal developments. The kinetics with which these transformed precursors (also referred to as colony-forming cells or CFC), appeared in two separate series of transplanted animals is outlined in Table 1. In one series of transfers, these precursors were not detected until the cells were passed into the tertiary recipient while in a second series, they were already present in the secondary recipient. Colonies from the methyl cellulose cultures were picked and expanded in liquid cultures. Immortalized, factor-dependent cell lines were derived from a number of them as detailed in Materials and Methods.

Table 1. Kinetics of Transformed Precursor Development in Transplanted Recipients

<table>
<thead>
<tr>
<th>Colony-Forming Cells per 10⁶ Spleen Cells</th>
<th>Mouse/Recipient</th>
<th>Time of Reconstitution (wk)</th>
<th>Transformed CFC</th>
<th>Normal CFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>src-27 (1)</td>
<td>10</td>
<td>1,000</td>
<td>140</td>
<td>410</td>
</tr>
<tr>
<td>src-27.1* (2)</td>
<td>4</td>
<td>2,500</td>
<td>230</td>
<td>350</td>
</tr>
<tr>
<td>src-27.1** (3)</td>
<td>2</td>
<td>625</td>
<td>230</td>
<td>625</td>
</tr>
<tr>
<td>src-31 (1)</td>
<td>15</td>
<td>410</td>
<td>230</td>
<td>410</td>
</tr>
<tr>
<td>src-31.2* (2)</td>
<td>4</td>
<td>2,500</td>
<td>230</td>
<td>350</td>
</tr>
<tr>
<td>src-31.1* (2)</td>
<td>4</td>
<td>250</td>
<td>230</td>
<td>250</td>
</tr>
<tr>
<td>src-31.1** (3)</td>
<td>2</td>
<td>&gt;5,000</td>
<td>625</td>
<td>625</td>
</tr>
</tbody>
</table>

Spleen cells (5 × 10⁶) were passaged at each transfer to recipients that had received 950 R irradiation. Colonies were grown in methyl cellulose cultures as described in Materials and Methods. Both normal and transformed CFC grew only in cultures which contained factors. The numbers presented are from those cultures that contained IL-3/IL-1 and Epo.

1 Transformed CFC are those which generate colonies consisting of only undifferentiated cells.

2 Normal CFC refers to all precursors that respond to the above combination of factors and includes pluripotential ones as well as those committed to the erythroid, neutrophil, macrophage, and mast cell lineages.
Growth characteristics of the v-src immortalized lines. The initial observation that the abnormal precursors generated colonies in methyl cellulose cultures in response to IL-3 and Epo or only Epo suggested that these cells were dependent on these factors for proliferation. To further investigate this possibility, we analyzed the growth characteristics of two cell lines (E4 and E31) in the presence or absence of Epo or IL-3. Figure 1 shows that both cell lines grew exponentially in the presence of either factor. In the absence of these factors E31 cells ceased to proliferate and finally disintegrated while E4 cells grew at a much reduced rate, always containing a high percentage (>40%) of nonviable cells. Both lines also showed a rapid proliferative response to these factors as determined by a short-term [3H]-thymidine incorporation assay. Within 48 hours, Epo stimulated thymidine incorporation sixfold to sevenfold in E4 cells and approximately 30-fold in E31 cells (Fig 1B). The response to IL-3 was much less dramatic, with a stimulation of 3- to 10-fold for each of the cell lines. A number of other lines including E19, E25, and E45 were also tested and found to respond to Epo to varying degrees in this proliferation assay (not shown).

The dependence on Epo for growth varied to some extent amongst the lines. E31 showed the overall strongest dependence, while lines E25 and E45 were similar to E4 in their proliferative response to Epo. Line E19 was only marginally Epo dependent and in fact able to proliferate in absence of any added factor at high densities (see below). These findings indicate that these cell lines require Epo or IL-3 for proliferation, with the strongest response being observed in the presence of Epo.

Differentiation phenotype of the v-src immortalized cell lines. The fact that many of the altered precursors required Epo for growth strongly suggested that they were of the erythroid lineage. To further define their lineage relationship and state of maturation, lines E4 and E31 were analyzed for hemoglobin content, for the presence of several lineage specific histone H5, and for the presence of several lineage specific cell surface antigens.

To demonstrate the presence of hemoglobin, the cells were stained with acid benzidine. Figure 2A shows that 10% to 30% of E31 and E4 cells were benzidine positive over a period of 20 days of culture, provided that Epo was present. The fact that the hemoglobin-positive population persisted for a 20-day culture period indicates that they are part of the immortalized population and not contaminating cells from the methyl cellulose cultures. Cells grown in IL-3 or cultured for short periods of time in the absence of factor, ceased to express hemoglobin (Fig 2A). However, when cultured again in the presence of Epo, they rapidly regained expression (0.1% benzidine-positive cells increasing to 17%), suggesting that the cells required this factor for expression of a partially differentiated phenotype as well as for proliferation. To determine what type of hemoglobin the v-src immortalized lines were producing, two different lines (E19 and E25) were analyzed by polymerase chain reaction for the presence of embryonic and adult globin RNA. Both were found to contain only adult globin RNA (data not shown).

We next attempted to induce differentiation of the benzidine negative fraction by treatment with various chemicals to demonstrate that the majority of cells within the E4 and E31 populations are of the erythroid lineage. Neither E4 nor E31 showed any significant differentiation response to DMSO whereas greater than 80% of the control Friend erythroleukemia cells became hemoglobin-positive by the same treatment (Fig 2B). The frequency of hemoglobin-positive E31 cells did, however, increase to more than 65% after a 2-day treatment with butyric acid, indicating that most cells within this population are of the erythroid lineage (Fig 2C).

To further substantiate this finding, E31 and E4 cells were stained with an antisera directed to the erythroid-specific avian histone H5. Pilot experiments had shown that this antisera cross-reacts with a nuclear antigen in
mouse erythroleukemia cell lines (H.B., unpublished observations). To establish that this antigen was indeed erythroid-specific, avian erythroblasts (positive control) as well as mixtures of Friend erythroleukemia cells and WEHI-3B cells were stained with the antibody. Virtually all the avian erythroblasts (Fig 3A) and the Friend erythroleukemia cells displayed distinct nuclear staining (Fig 3B and data not shown). No staining was observed in the WEHI-3 cells. When stained with the anti-H5 antiserum, between 80% and 90% of the E4 and E31 cells showed a distinct nuclear fluorescence. (Fig 3, C and D). Taken together with the above results, these findings indicate that most, if not all, cells within the E4 and E31 populations are of the erythroid lineage.

Cells from eight lines (E4, E13, E19, E25, E31, E40, EP21, and EP25) were also stained with a panel of antibodies directed against lineage-specific surface antigens. All these lines showed the same pattern of staining. They did not stain with M1/70, RB6-8C5, T.24, anti-B220, and E13 161-7 (anti-Sca-1), which detect determinants on macrophages, neutrophils, T cells, pre-B and B cells, and early precursors and stem cells, respectively. They all stained with the antibody J11D, which detects an antigen found on the majority of bone marrow cells including those belonging to the erythroid lineage. These findings are consistent with the above observations indicating that the v-src transformed populations are of the erythroid lineage.

Recent studies have shown that the erythroid defect in W mice results from mutations in the c-kit proto-oncogene that encodes a receptor with tyrosine kinase activity, suggesting that this receptor is involved in normal erythropoiesis. If the v-src immortalized cell lines represent normal erythroid precursors that are blocked in their differentiation pathway, they should express the c-kit gene. Figure 4 shows that three different lines tested, E19, E31, and E45, all contain readily detectable levels of c-kit message. More recent studies have shown that E19 also contains c-kit-specific tyrosine kinase, indicating the presence of a functional protein (W. Alexander, unpublished observation). Control WEHI-3B cells as well as a v-src transformed adherent marrow cell line, BM.AD-src, did not contain detectable levels of c-kit message. The absence of c-kit message in the BM.AD-src cells which express v-src (see below) indicate that c-kit expression in the erythroid populations is not a result of v-src expression.

Clonality of the immortalized populations. To determine the clonal relationship of these cell lines to each other as well as to hematopoietic cells found in the spleens of the different recipients, we analyzed the proviral integration site in the DNA of the various populations. Figure 5A shows that the DNA from the spleen of primary recipient src-31 contains one predominant band plus numerous smaller ones. We interpret this pattern as indicating the presence of one dominant large clone plus a number of smaller clones. Spleens from the secondary and tertiary recipients contained the predominant clone plus two other clones (or possibly one clone with two integration sites) of comparable size. These two additional clones were present in the primary recipients at much smaller size and have obviously increased in size during the transplantation. Three cell lines, E19, E25, and E27, were found to consist of the same clones that were present in the spleens of these recipients. In a subsequent analysis, E31 and E45 were also found to contain the identical clones (data not shown). The findings are essentially similar in the second series of transfers. The erythroid populations EP21 and EP28 were found to contain the same clones present in the spleen (src-27.1**) from which they were derived. Identical clonal
was also expressed in these cells we analyzed them for the presence of the appropriate mRNA as well as for pp60 kinase activity. Two different lines tested, E19 and E25, contained the three expected v-src-specific transcripts, indicating that the gene was being expressed (data not shown). In addition, readily detectable levels of pp60 kinase activity were present in E31, E19, as well as in the src transformed adherent marrow line, BM.AD-src and in the N-TK-src virus producing line (GP+E.N-TK-src) (Fig 6). No activity was detected in the control GP+E.N2 cells which produce the N2 neo expressing virus. These findings show that the v-src oncogene is efficiently expressed in the immortalized erythroid lines.

Restricted factor dependence of the immortalized erythroid lines. The previous observations indicate that the immortalized cell lines are of the erythroid lineage and maintain responsiveness to their normal regulators, namely Epo and IL-3. We were next interested in determining whether these cells would grow in response to a number of other hematopoietic growth factors. To be able to quantify this response, cells were initially tested for their ability to grow in methyl cellulose cultures in response to Epo. Figure 7A shows that approximately 30% of the E31 population grew and formed large colonies in the presence of Epo. These colonies were similar in morphology to the original colonies isolated from the cells of the reconstituted mice. The linear dose response obtained (slope = 1) indicates that the
colonies arose from cells responding to Epo and that other growth factors were not required. Occasionally, small numbers of colonies developed in the absence of factors. However, this response was always less than 5% of the response to Epo.

E31 cells were next plated in methyl cellulose cultures in the presence of a variety of other hematopoietic growth factors. Both MGF (stem cell factor, SCF; Kit ligand, KL), the ligand to the c-kit encoded receptor, and Epo stimulated large numbers of colonies. The response to IL-3 was significantly less, approximately 40% of the response to Epo. Surprisingly, the cells also consistently responded to GM-CSF. However, they did not respond to M or G-CSF or to IL-1, IL-4, or IL-6. This finding indicates that the E-31 cells, although immortalized, show a growth factor responsiveness similar to normal erythroid precursors.

The morphology of the colonies grown in the presence of various growth factors did not differ dramatically. Those grown in the presence of Epo and MGF tended to be larger than those grown in the presence of IL-3 or GM-CSF. Occasionally, a small proportion of the colonies (≤5%) developed clusters of cells that resemble colonies derived from normal erythroid precursors. These were present only in cultures that contained Epo.

Isolation of factor-independent lines. Although all of the cell lines grew in response to Epo, slow growth in absence of factor, particularly at high-cell density, was observed in almost all of them. Provided that the cells were maintained at a high density, factor-independent populations could be generated from most lines. To determine if a vigorously growing factor-independent line could be established, cells from E19 were passaged repeatedly in the absence of factor. After the initial removal of factors, approximately 50% of the cells died and the remainder grew slowly, provided they were maintained at relatively high densities. Following several weeks of growth in the absence of factor, the cells were plated in methyl cellulose and a number of colonies picked and expanded in liquid culture. The resulting lines grew well in the absence of factor and were less density dependent than the parental line. The growth characteristic of one of these factor-independent lines, E19.10, is compared with the growth of the parental line (E19) in Fig 8. In the absence of Epo, the parental cells formed only a small number of colonies at high cell densities while growing efficiently at all densities in the presence of the factor (Fig 8A). In contrast, E19.10 generated large numbers of colonies in the presence and absence
of Epo when seeded at high density. Colony development by E19.10 cells was strongly density dependent, suggesting that they had become dependent on autocrine factors for growth. Despite the fact that they had been maintained in the absence of factor for more than 6 months, the E19.10 cells did retain some responsiveness to Epo.

To test whether or not E19.10 cells produced factor(s) that they respond to, their conditioned medium was included in the methyl cellulose cultures with them or with the parental cells. As shown in Fig 8, E19.10 conditioned medium (CM) stimulated the growth of both populations at all cell densities. The fact that the CM overcame the cell density requirement for growth of the E19.10 population indicates that these cells are producing a growth factor that they can respond to. In addition to stimulating the growth of these populations, the E19.10-CM also stimulated the growth of normal precursors of the erythroid, neutrophil, macrophage and mast cell lineages (Table 2). The development of the erythroid colonies was dependent on the addition of Epo. Colonies from the other precursors developed in the presence of only the CM. Conditioned medium from the parental population (E19-CM) contained no such colony-stimulating activity.

The broad spectrum of precursors stimulated by the E19.10 CM suggested that the cells were producing IL-3. However, Northern analysis indicated that the cells contained very low amounts, if any, IL-3-specific mRNA. This was true for untreated populations as well as for cells stimulated with Epo or pretreated with cycloheximide (data not shown). Despite this indication that the cells were not producing significant amounts of IL-3, a neutralizing antibody to this factor did inhibit all of the erythroid colony-stimulating activity in the E19.10 supernatant (Table 2). The only colonies that persisted in these cultures consisted of neutrophils and macrophages. The antibody also neutralized the activity in E19.10 CM that stimulated colony growth of E31 cells (data not shown). These findings suggest that the erythroid colony-stimulating activity in E19.10-CM is IL-3 or a factor that is structurally related to it. They also indicate that the cells are producing factors in addition to IL-3 that can stimulate the growth of neutrophil and macrophage colonies and are not inhibited by the antibody.

**DISCUSSION**

In this report we describe the development of transformed erythroid precursors in mice transplanted with spleen cells expressing the v-src oncogene. These transformed precursors are blocked in their developmental potential and were able to generate immortalized lines in culture in the presence of specific growth factors. This is the first demonstration that the v-src oncogene can transform mouse erythroid precursors.

The cell lines described in this report differ, in several aspects, from the immortalized cells lines generated from long-term bone marrow cultures infected with a v-src-containing virus. First, the lines we have established

**Table 2. Colony-Stimulating Activity in E19.10 CM**

<table>
<thead>
<tr>
<th>Factor*</th>
<th>Epo†</th>
<th>Anti-IL-3‡</th>
<th>Colonies per 10⁶ Cells</th>
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<tbody>
<tr>
<td>IL-3</td>
<td>+</td>
<td>–</td>
<td>E 144</td>
</tr>
<tr>
<td>E19.10-CM</td>
<td>+</td>
<td>–</td>
<td>85 84</td>
</tr>
<tr>
<td>E19.10-CM</td>
<td>–</td>
<td>–</td>
<td>0 127</td>
</tr>
<tr>
<td>E19-CM</td>
<td>+</td>
<td>–</td>
<td>0 0</td>
</tr>
<tr>
<td>IL-3</td>
<td>+</td>
<td>+</td>
<td>0 0</td>
</tr>
<tr>
<td>E19.10-CM</td>
<td>+</td>
<td>+</td>
<td>64</td>
</tr>
</tbody>
</table>

Bone marrow cells from CBA/J mice were plated in methyl cellulose cultures as described in Materials and Methods. E are pure erythroid colonies and GM/MAST represent colonies of pure neutrophils, pure macrophages, pure mast cells, and mixed neutrophil and macrophage.

* Cultures contain either 10 U of IL-3 or 40% E19.10 or E19 CM.
† Where indicated, cultures contain 2 U of Epo.
‡ Where indicated, the cultures contain a 1/20 dilution of a culture supernatant from hybridoma cells which produce the anti-IL-3 antibody. The antibody is specific for IL-3 as it does not inhibit colony formation by other growth factors such as GM-CSF (data not shown).
were derived from precursors that are committed to the
erythroid lineage and are able to respond to the factors that
regulate normal erythropoiesis. Those generated from in-
fected long-term marrow cultures appear to represent cells
at an earlier stage of hematopoietic development, as they
are able to give rise to differentiated progeny of multiple
lineages.\(^{29}\) Second, all of the erythroid lines that we have
tested contain the recombinant virus (N-TK-src) and ex-
press the v-src oncogene, suggesting that it is acting directly
on these cells and is required for maintenance of the
transformed phenotype. In contrast, those derived from the
infected marrow cultures do not contain the v-src–
expressing virus, indicating that the v-src gene plays an
indirect role in this development.

The erythroid lines immortalized by the v-src oncogene
differ from most other mouse erythroleukemia cell lines in
that they can be maintained as factor-dependent popula-
tions. Even a “factor-independent” subpopulation re-
sponded to Epo following long periods of growth in the
absence of factors (Fig 8). In contrast, most other estab-
lished erythroid lines grow in the absence of specific growth
factors.\(^{30-41}\) The fact that the v-src transformed erythroid
cells do remain factor dependent has allowed us to deter-
mine their responsiveness to a broad spectrum of growth
factors. The results obtained from this analysis indicate that
these immortalized cells respond to the same factors that
stimulate the growth of normal erythroid precursors; namely
MGF, Epo, and IL-3. The relatively strong response to
GM-CSF may be abnormal or it might reflect a particular
stage of erythroid development that is responsive to GM-
CSF as it has been reported that some erythroid precursors
do respond to this factor.\(^{41}\)

From their pattern of responsiveness to Epo and IL-3,
one would position the v-src immortalized cells at the
transition point between BFU-E and the CFU-E within the
erythroid lineage. The presence of large amounts of c-kit
RNA in these cells and their responsiveness to the ligand
MGF would also be consistent with this interpretation.
Although the c-kit receptor is likely to be involved in a
number of different stages of hematopoietic development,
data from a recent study would suggest that it plays a key
role at the stage between the BFU-E and the CFU-E.\(^{41}\)

Another unique feature of the v-src immortalized lines is
that they could not be induced to differentiate in the
presence of DMSO whereas most other mouse erythroleu-
kemia lines do differentiate to some extent in the presence
of this drug.\(^{31}\) The v-src immortalized lines did, however,
differentiate in the presence of butyric acid and in this
respect are similar to v-erb-B transformed avian erythro-
blasts.\(^{41}\)

Although the erythroid lines could be maintained as
factor-dependent populations, it was possible to establish
factor independent subpopulations from them. At least one
of these factor-independent lines (E19.10) appears to grow
via an autocrine mechanism as its growth is cell density-
dependent and its own conditioned medium stimulated its
growth. The factor(s) present in this CM also stimulated a
broad spectrum of normal precursors including a high
proportion of those from the erythroid lineage. Although
the cells contain very little IL-3 specific mRNA, the fact
that all of the erythroid colony-stimulating activity could be
inhibited with a neutralizing antibody to IL-3 strongly
suggests that it is the active factor in the supernatant. The
E19.10 cells are clearly producing other factors as the
antibody was unable to inhibit all of the neutrophil and
macrophage colony-stimulating activity. The nature of this
colony-stimulating activity as well as the other types of
factors produced by these cells remains to be determined.

A number of other studies lend support to the notion that
expression of the v-src oncogene can lead to growth via an
autocrine mechanism. Adkins et al\(^{46}\) found that expression
of v-src in factor-dependent \(m b\) transformed myeloblasts
led to the development of a population of cells which
produced the factor that they required for growth. More
recently, Anderson et al\(^{49}\) showed that infection of an
IL-3–dependent cell line (32DCl3) with a v-src–expressing
virus could abrogate their requirement for growth factor.
These factor-independent v-src expressing cells were found
to produce a factor which stimulated the growth of normal
32D c13 cells.

Our findings outlined in this report are consistent with
earlier studies in the avian system which have demon-
strated that expression of the v-src oncogene can transform chicken
erthyroid precursors both in vitro and in vivo.\(^{40}\) However,
transformation of erythroid precursors in the avian system
is rapid, occurring within several weeks of infection. In
mice, transformed cells were not observed until spleens
from primary reconstituted recipients were serially trans-
planted to secondary and tertiary animals, spanning a period
of 16 to 20 weeks after the initial infection. The extended
period of time required for the appearance of these
transformed cells would indicate that events in addition to
expression of the v-src oncogene are required for their
development and would be consistent with the general
hypothesis that the transformed phenotype results from
multiple genetic changes. One gene that could be involved
in the development of these transformed populations is the
p53 proto-oncogene as disruption of this gene appears to be
associated with the establishment of most Friend virus
transformed erythroid lines.\(^{47,48}\) A preliminary analysis has
indicated that some of the v-src transformed erythroid lines
do have rearranged p53 genes. Whether all of the estab-
lished lines have mutations at this locus remains to be
determined. Another locus that appears to be involved in
the development of erythroleukemia following Friend virus
infection is \(s p l-1.\)\(^{49}\) Whether it is also involved in the
establishment of the transformed erythroid population is
currently under investigation.

In conclusion, the data presented in this report show that
prolonged expression of the v-src oncogene in mouse
hematopoietic cells in vivo leads to the emergence of
transformed erythroid precursors. These precursors are
unique in that they were able to generate factor-dependent
cell lines under the appropriate conditions in culture. The
development of such factor-dependent erythroid lines pro-
vides an ideal system with which to search for novel
regulators of erythropoiesis. In addition, the presence of the same clonal markers in the cell lines and in the hematopoietic tissues of the various transplanted recipients provides a model system for analyzing and defining the stage of the various molecular changes that ultimately contributed to the final transformed phenotype.

REFERENCES

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Factor-dependent erythroid cell lines derived from mice transplanted with hematopoietic cells expressing the v-src oncogene

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