The Growth of Rauscher Erythroleukemia Cells Is Mediated by Autocrine Production of a Factor With Biological Activity Similar to Interleukin-3

By G. Migliaccio, A.R. Migliaccio, S. Ruscetti, and J.W. Adamson

Under serum-deprived and chemically defined culture conditions, the growth of Rauscher erythroleukemia cells is mediated by an autocrine mechanism. The growth-promoting activity is produced by fresh or irradiated cells and resembles the activity of interleukin-3 (IL-3) in its ability to sustain colony formation from three of four IL-3-dependent cell lines and to induce formation of granulocyte/macrophage (GM) colonies and, in the presence of erythropoietin (Ep), of erythroid bursts and mixed erythroid colonies. IL-3, IL-1, IL-4, IL-6, GM colony-stimulating factor (GM-CSF), G-CSF, M-CSF, Ep, and media conditioned by concanavalin A-stimulated mouse spleen cells or phytohemagglutinin-stimulated LBRM 33 cells were unable to induce proliferation of the Rauscher erythroleukemia cells.

The Rauscher erythroleukemia cell line was established from DBA mice that had been injected subcutaneously with liver cells from Rauscher virus complex-infected animals in the terminal phase of erythroleukemia. The cell line is composed of blast-like cells that induce tumors when injected into histocompatible recipients and that give rise to colonies in agar or in plasma clot cultures. In the latter case, 4% of the colonies contain cells that synthesize hemoglobin and are benzidine-positive. The cells also respond to erythropoietin (Ep) by increasing both the size of the colonies and the percent of benzidine-positive cells.

The mechanisms involved in the transformation process mediated by viruses that do not contain oncogenes, such as the Rauscher virus complex, have not been elucidated. By analogy with other models of tumorigenesis, the virus could either activate the production of a specific growth factor or increase the responsiveness of the cell to growth factors. Numerous examples of the former mechanisms have been described in both hematologic and nonhematologic systems (for recent reviews see references 3 and 4). The latter mechanism could be brought about either through the induction of a functionally altered growth factor receptor or by increasing the expression of a growth factor receptor. Activated as well as normal growth factor receptors expressed at a high level may lead to cell transformation. Examples of activated growth factor receptors are represented by the v-erb B and v-fms oncogenes, which are derived from the genes encoding epidermal growth factor (EGF) and colony-stimulating factor-1 (CSF-1 or M-CSF) receptors, respectively. An example of cell transformation by increased expression of a normal growth factor receptor is represented by NIH 3T3 cells transfected with the normal EGF receptor gene and that display increased EGF receptor numbers and form EGF-dependent transformation foci.

Recently, it has been reported that Rauscher erythroleukemia cells have approximately ten times more Ep receptors than do normal murine erythroid colony-forming cells (CFU-E). In order to elucidate the role exerted by the enhanced expression of Ep receptors on the growth of these cells, we have investigated their growth requirements in cultures deprived of fetal bovine serum (FBS), which may contain Ep. Our results indicate that the growth of the Rauscher erythroleukemia cells is independent of Ep but is dependent on another growth factor produced by the cells themselves. This growth-promoting activity does not correspond to any of the known hematopoietic growth factors. The results suggest that the acquisition of autocrine growth regulation by the leukemic cells represents the event that led to the establishment of the cell line.

MATERIALS AND METHODS

Mice

Male F1 hybrids (C57L/J × A/HeJ; Jackson Laboratories, Bar Harbor, ME) were used in this study. Mice (6 to 8 weeks old) were provided with sterilized pelleted food and sterilized, acidified water ad libitum.

Cell Lines

Two subclones, red 5-1.5 and 5-1.5a, of the Rauscher erythroleukemia cell line were obtained by subcloning the original line developed by De Boer et al1 in methylcellulose. The red 5-1.5 clone gives rise to benzidine-positive cells in cultures supplemented with Ep, while the red 5-1.5a does not, despite the fact that the number of Ep receptors and their affinity are the same on both subclones. These
lines express both Rauscher murine leukemia virus (MuLV) and spleen focus-forming virus (SFFV)-encoded proteins and also release into the supernatant high levels of reverse transcriptase activity and infectious virus that is capable of inducing erythroleukemia in adult NIH Swiss mice (S. Ruscetti, unpublished observations, 1988). The cells were passed twice a week in 75 cm² plastic flasks (Corning) containing RPMI (GIBCO, Grand Island, NY), β-mercaptoethanol (7.5 x 10⁻⁵ mol/L), antibiotics (100 U of penicillin, 250 ng of amphotericin B, and 100 μg of streptomycin) and either FBS (10% vol/vol; Hyclone, Logan, UT) or a mixture of nutrients (see below). The B6 SUA16 (a gift of Dr. J. Greenberger), Da-17 (a gift of Dr. L. Guilbert), 32D cl 23,11 and FDC-P113 (a gift of Dr. J. Pierce) cell lines were maintained by biweekly passage in McCoy’s medium (GIBCO) modified as described by Greenberger et al 14 and supplemented with antibiotics, t-glutamine, and pyruvic acid (1% vol/vol; GIBCO) and horse serum (10% vol/vol; Hyclone). Conditioned medium from phytohemagglutinin (PHA)-stimulated LBRM 33 cells15 (5% vol/vol) was also added as a source of interleukin-3 (IL-3).

Growth Factors

Medium conditioned by concanavalin A-stimulated spleen cells (SCM) or PHA-stimulated LBRM 33 cells was prepared under serum-deprived conditions as described.14 The pure growth factors studied included murine IL-327 and granulocyte-macrophage CSF (GM-CSF)18 (J.J. Mermod; Glaxo, Basel, Switzerland), human Ep19 and G-CSF28 (Amgen, Thousand Oaks, CA), human IL-129 (Immune, Seattle) and IL-622 (Genetics Institute, Cambridge, MA), murine IL-423 (DNAX, Palo Alto, CA), murine M-CSF24 (Dr R. Stanley), and bovine insulin (Sigma, St Louis). Each conditioned medium or growth factor was used at a concentration previously shown to be maximally effective in promoting colony formation by purified murine progenitor cells under FBS-deprived conditions.25,26

Medium for Serum-Deprived and Chemically Defined Cultures

Serum-deprived cultures. The Rauscher erythroleukemia cell line was cultured in the presence of bovine serum albumin (BSA; insulin-free for radioimmunoassay; 5 x 10⁻⁴ mol/L), BSA-adsorbed cholesterol (4 μg/mL) and soybean lecithin (12 μg/mL), and iron-saturated human transferrin (5 x 10⁻⁷ mol/L). All the other cell lines as well as normal progenitors required a more complex mixture of nutrients to grow under FBS-deprived conditions including BSA (2 x 10⁻⁴ mol/L), BSA-adsorbed cholesterol (4 μg/mL) and soybean lecithin (12 μg/mL), iron-saturated human transferrin (5 x 10⁻⁷ mol/L), bovine insulin (1.7 x 10⁻⁸ mol/L), nucleosides (10 μg/mL each), inorganic salts, sodium pyruvate (10⁻⁴ mol/L), and t-glutamine (2 x 10⁻⁴ mol/L) as previously reported.27 All the reagents were from Sigma and were prepared as described.27

Chemically defined protein-free cultures. The FBS was substituted by cholesterol (4 μg/mL) and soybean lecithin (12 μg/mL) (both dissolved in ethanol), nucleosides (10 μg/mL each), inorganic salts, sodium pyruvate (10⁻⁴ mol/L), and t-glutamine (2 x 10⁻³ mol/L).

Colony Assay From Normal Marrow Progenitor Cells or From Cell Lines

Normal GM colony and erythroid burst growth was assessed in semisolid medium containing the following components in Iscove’s modified Dulbecco’s medium (IMDM): methylcellulose (0.8% wt/vol, final concentration), β-mercaptoethanol (7.5 x 10⁻⁵ mol/L), and the mixture of components described above which replaced FBS.

The source of progenitor cells was either a single cell suspension of tibial marrow (5 x 10⁶ cells/mL) or purified progenitor cells (500 cells/mL; see below). Colony formation from cell lines was obtained in semisolid cultures in which the methylcellulose was replaced by agar (Bacto Agar, 0.6% wt/vol; GIBCO). In some experiments, a double 1 mL agar layer was used in which the marrow cells were cultured in the upper layer and irradiated Rauscher cells were included in the lower layer. The dishes were kept for ten minutes at 4°C between the formation of the two layers.

Scoring Criteria

Colonies from normal bone marrow or cell lines were scored at day 8 of culture. Erythroid bursts and mixed erythroid colonies contained ≥1.5 x 10⁵ cells per colony; GM colonies contained ≥5 x 10⁵ cells per colony, and colonies from cell lines contained at least 5 x 10⁶ cells per colony.

Purification of Hematopoietic Progenitors

Murine progenitors were purified by collecting blast cell colonies (bce) in primary cultures of spleen cells harvested from 5-fluorouracil-treated mice.23,24 Mice, injected intravenously (IV) with 5-fluorouracil in saline (150 mg/kg body weight), were killed four days later and the spleens were removed aseptically. Cells (2 x 10⁶/mL) were plated in 35-mm dishes containing 1 mL of the semisolid medium described above. The cultures were supplemented with SCM (5% vol/vol). Bcc were detected by day 7 of culture. Individual colonies were collected with a thin Pasteur pipette and pooled. The pool of bce-derived cells was washed twice with fresh medium and the cells plated at a concentration of 250 to 500 cells/mL in secondary serum-deprived cultures. Bcc-derived cells gave rise to secondary colonies with a cloning efficiency of 30% to 70% in FBS-deprived cultures supplemented with SCM and Ep.

Cell Irradiation

The Rauscher erythroleukemia cells were irradiated with 3,000 rad with a 137Cesium Mark I cell irradiator, model 32 (JL Shepherd, Glendale, CA). The viability of the cells after irradiation was evaluated by trypan blue dye-exclusion. Only 2% to 5% of the irradiated cells were trypan blue positive. This percent increased to 10% to 20% after eight days of culture. The irradiated cells failed to give rise to colonies in semisolid cultures.

Preparation of Medium Conditioned by Rauscher Erythroleukemia Cells, of Cell Membranes, and of Glutaraldehyde-Fixed Cells

Supernatants were collected from serum-deprived flasks after four days (Fig 1) and tested for the presence of CSA either directly or after being concentrated 20-fold with a Micro-ConFlt apparatus through a PA-ProDiMem MCF-10 membrane (Bio-Molecular Dynamics, Beaverton, OR). Cell membranes were prepared by three cycles of freezing and thawing. The membranes were collected by centrifugation and resuspended in fresh medium. A volume of membrane preparation equivalent to 2 to 5 x 10⁶ cells was added per milliliter of culture. Rauscher cells also were fixed with glutaraldehyde as described26 and 2 x 10⁶ cells were added to 1 mL of culture.

1H-Thymidine Incorporation

Rauscher erythroleukemia cells (2.5 x 10⁶ or 2.5 x 10⁷ cells/mL; 200 μL/well) were cultured overnight in FBS-supplemented, FBS-deprived, or chemically defined medium in 24-well dishes (Falcon). Each data point was obtained in quadruplicate. The cells were
Incubated for a period of four hours with 1 μCi ³H-thymidine (³H-TdR) per well (Amersham, Arlington Heights, IL; specific activity 25 Ci/mmol) or ³H-TdR plus a tenfold excess of cold TdR. The cells were then transferred to a glass fiber filter (Mash II; MA. Bioproducts, Walkersville, MD). Each filter was washed three times with 3 mL of cold tricloracetic acid (TCA; 10% vol/vol) and then once with cold ethanol (70% vol/vol). The filters were allowed to dry and then were immersed in scintillation fluid for counting.

**Northern Analysis of Hematopoietic Growth Factor Expression from Rauscher Erythroleukemia Cells**

RNA was extracted from cells growing in log phase by the guanidinium-isothiocyanate method. PolyA-RNA was selected by affinity chromatography on oligo(dT)-cellulose (Collaborative Research, Lexington, MA). PolyA-selected and total RNA were separated by electrophoresis on a 1.2% agarose gel in the presence of 6% formaldehyde, and transferred onto a nitrocellulose filter, which was baked at 85°C before the hybridization. All the procedures were carried out according to standard protocols. The filters were exposed overnight at −70°C in the presence of an intensifying screen. Exposure times were altered as required.

**Assay of Murine IL-6**

Cell supernatants were assayed for IL-6 by R. Nordan (National Cancer Institute, Bethesda, MD) with a sensitive bioassay using either an IL-6-dependent murine B cell hybridoma line, B9.4, or an IL-6-dependent plasmacytoma cell line (T1165.85.2.1.35). The assay using the B9 cells will detect as little as 1 pg of IL-6/mL.

**RESULTS**

**Growth of Rauscher Erythroleukemia Cells in Serum-Deprived or in Chemically Defined Cultures**

The nutrients required for the growth of Rauscher erythroleukemia cells in the absence of FBS were evaluated in a series of experiments summarized in Figs 1 and 2 and in Table 1. The criteria we used to define optimal cell growth included the growth curve, the amount of ³H-TdR incorporated under different culture conditions, and the capacity of
the cells to be propagated under such conditions. No differences were observed between the two Rauscher erythroleukemia subclones and, consequently, the results have been pooled.

The growth curves of the Rauscher erythroleukemia cells in FBS-supplemented or serum-deprived and chemically defined cultures were similar when the cells were plated at 2.5 × 10⁶ cells/mL (Fig 1). The cell doubling time was 12 and 18 hours in FBS-supplemented and in FBS-deprived cultures, respectively, and the cell saturation density (2.7 × 10⁶ cells/mL) was the same for all the cultures (Fig 1). The cells were able to be passed for more than 1 month in each culture condition (approximately eight passages).

Table 1 compares the ³H-TdR incorporation in the TCA-precipitable fraction of Rauscher erythroleukemia cells in FBS-supplemented, in FBS-deprived, and in chemically defined cultures at different cell concentrations. At a concentration of 2 × 10⁵ cells/mL, no significant differences in ³H-TdR incorporation were detected among the different cultures. Since the cell line grew at this cell concentration as defined cultures at different cell concentrations. At a concentration of 2.5 × 10⁶ cells/mL, the growth was not dependent on the presence of BSA, transferrin, or insulin. The only parameter identified that affected cell growth under stringent culture conditions was the number of cells at the inoculum. After overnight incubation in serum-free or protein-free medium at a cell concentration of 2.5 × 10⁶ cells/mL, no significant differences in ³H-TdR incorporation were detected among the different cultures. Since the cell line grew at this cell concentration as defined cultures at different cell concentrations. At a concentration of 2.5 × 10⁶ cells/mL, the growth was not dependent on the presence of BSA, transferrin, or insulin. The only parameter identified that affected cell growth under stringent culture conditions was the number of cells at the inoculum. After overnight incubation in serum-free or protein-free medium at a cell concentration of 2.5 × 10⁶ cells/mL, the cells did not incorporate ³H-TdR (Table 1). Proliferation at this cell concentration was not initiated by insulin (Table 1). Ep (Fig 1), or SCM (data not shown).

As shown in Table 2, Rauscher erythroleukemia cells gave rise to colonies when cultured in methylcellulose in the presence of FBS and with a cloning efficiency of 40%. At a concentration of 500 cells/mL, Rauscher erythroleukemia cells did not form colonies in FBS-deprived cultures. Irradiated Rauscher erythroleukemia cells did not form colonies under any conditions but the majority remained viable, since only 10% to 20% took up trypan blue after eight days in suspension culture. When unirradiated Rauscher cells (500/mL) were cocultured with irradiated cells under FBS-deprived conditions, colony growth was restored (Table 2, Fig 2). Maximal colony growth was observed with 2 × 10⁵ irradiated cells (Fig 2). The same results were seen when the irradiated cells were separated from the target cells by an agar layer (Fig 2).

Several sources of hematopoietic growth factors were used in an attempt to stimulate the growth of the Rauscher cells in FBS-deprived cultures (Table 2). GM-CSF, G-CSF, Ep, IL-3, IL-1, IL-4, IL-6, and M-CSF, at concentrations that were optimal for the growth of enriched murine progenitors in serum-deprived cultures (3) (unpublished observations), failed to stimulate Rauscher cell colony growth (Table 2). Also ineffective were PHA-LBRM 33 conditioned medium, SCM, conditioned medium from the Rauscher cells themselves, Rauscher cell membrane preparations, or glutaraldehyde-fixed cells (Table 2).

Effect of Rauscher Erythroleukemia Cells on the Proliferation of IL-3-Dependent Cell Lines and on the Formation of Hematopoietic Colonies From Purified Murine Progenitor Cells

To characterize the activities produced by the Rauscher erythroleukemia cells, we investigated the ability of irradiated Rauscher cells to induce colony formation by IL-3-dependent cell lines under FBS-deprived conditions. Four different cell lines were studied: B6SUTA, 32D cl 23, DA-1, and FDC-P1. The results are summarized in Table 3. Irradiated Rauscher cells induced colony formation by 32D cl 23, DA-1, and FDC-P1 cells. The number of colonies induced was comparable with the numbers seen in the IL-3-stimulated controls for FDC-P1 cells, 80% of the

Table 1. The Effect of Cell Concentration and the Presence of FBS on the ³H-Thymidine Incorporation by Rauscher Erythroleukemia Cells

<table>
<thead>
<tr>
<th>Number of Cells/mL</th>
<th>CPM (× 10³)/Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FBS+</td>
</tr>
<tr>
<td>2.5 × 10⁴</td>
<td>³H-TdR</td>
</tr>
<tr>
<td>2.5 × 10⁵</td>
<td>³H-TdR + cold TdR</td>
</tr>
<tr>
<td>2.5 × 10⁶</td>
<td>³H-TdR</td>
</tr>
<tr>
<td>3H-TdR + cold TdR</td>
<td>14 ± 1</td>
</tr>
</tbody>
</table>

*Insulin was added to a final concentration of 1.7 × 10⁻⁶ mol/L.
†The results are expressed as the mean ± SD of three separate experiments performed in quadruplicate.

Table 2. Cloning of Rauscher Cells in FBS-Deprived Cultures Stimulated With Different Growth Factors

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Stimulus Added</th>
<th>Colonies/500 Cells Plated</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS+</td>
<td>None</td>
<td>186 ± 34</td>
</tr>
<tr>
<td>FBS+</td>
<td>Irradiated Rauscher cells*</td>
<td>170 ± 48</td>
</tr>
<tr>
<td></td>
<td>Rauscher cell CM*</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Glutaraldehyde-fixed</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Rauscher cells*</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ep (3 U/mL)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>G-CSF (10 U/mL)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M-CSF (100 U/mL)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>GM-CSF (10⁴ U/mL)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IL-1 (10⁴ U/mL)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IL-3 (10⁴ U/mL)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IL-4 (30 U/mL)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IL-6 (20 U/mL)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Irradiated or glutaraldehyde-fixed Rauscher cells were plated at a concentration of 2 × 10⁶ cells/mL. A volume of 25 µL of Rauscher cell membrane preparation, corresponding to the membranes derived from 2 × 10⁶ cells, was added. Rauscher cell CM was added at concentrations up to 50% vol/vol.
control value for the 32D cl 23 cells, and 20% for the DA-1 cells. It is possible that irradiated Rauscher cells released a transforming activity that induced growth factor independent growth of these cell lines. This possibility was excluded since 32D cl 23 cells grown in the presence of irradiated, Rauscher cells did not grow when subsequently transferred into cultures not supplemented with IL-3 (results not shown).

The activity of the Rauscher erythroleukemia cells was further characterized by investigating the effect of irradiated cells on normal murine GM colony and erythroid burst growth. Irradiated Rauscher cells sustained GM colony formation in FBS-deprived cultures of normal marrow cells (Fig 3). At the highest Rauscher cell concentration tested (2 × 10^5/mL), the number of GM colonies observed was 75% of the number observed in SCM-stimulated cultures. The colonies were picked, spread on glass slides, and the cells stained with May-Grunwald/Giemsa. Seventy percent of the cells were macrophages, and the remainder were granulocytes.

Since irradiated Rauscher cells still release virus as measured by the reverse transcriptase assay (data not presented), we analyzed the normal progenitor cells that had been cocultured with the irradiated Rauscher erythroleukemia cells for the presence of viral proteins by indirect immunofluorescence using a goat anti-Rauscher MuLV-gp70 serum (from the Division of Cancer Etiology, National Cancer Institute). No fluorescence-positive cells were observed with the anti-gp70 serum, and the growth-promoting effect of the irradiated Rauscher cells was not inhibited by the antiserum (data not shown).

Irradiated cells were still able to induce GM colony growth when separated from the target cells in a two layer agar system (Fig 3). However, supernatants from the Rauscher erythroleukemia cells were unable to stimulate GM colony growth under these conditions. Rauscher erythroleukemia cell membrane preparations or glutaraldehyde-fixed cells were also ineffective in promoting normal progenitor cell growth (data not shown).

To exclude the possibility that the CSA of irradiated Rauscher cells was mediated indirectly by activation of accessory cells, we tested the effect of the irradiated cells on the growth of bcc-derived cells. The results are shown in Table 4. Irradiated Rauscher cells induced GM colony formation and, in the presence of Ep, erythroid bursts and mixed erythroid colonies in these cultures.

**Expression of Hematopoietic Growth Factors by the Rauscher Erythroleukemia Cells**

RNA was extracted from Rauscher erythroleukemia cells growing in log phase or 24 hours after irradiation and probed for the presence of known hematopoietic growth factor transcripts. In both poly A-enriched or total RNA preparations, multiple hybridization bands were seen when the gel

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**Table 3. Effect of Irradiated Rauscher Erythroleukemia Cells on the Growth of IL-3-Dependent Murine Cell Lines in FBS-Deprived Cultures**

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Stimulus Added</th>
<th>B6 SUA (± SD)/500 Cells</th>
<th>32D cl 23 (± SD)/500 Cells</th>
<th>DA-1 (± SD)/500 Cells</th>
<th>FDC-P1 (± SD)/500 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS*</td>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6 ± 5</td>
</tr>
<tr>
<td></td>
<td>IL-3</td>
<td>317 ± 43</td>
<td>110 ± 16</td>
<td>64 ± 12</td>
<td>179 ± 60</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FBS</td>
<td>IL-3</td>
<td>310 ± 40</td>
<td>207 ± 20</td>
<td>47 ± 6</td>
<td>143 ± 12</td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td>—</td>
<td>0</td>
<td>3 ± 1</td>
<td>93 ± 6</td>
</tr>
<tr>
<td></td>
<td>M-CSF</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>G-CSF</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ep</td>
<td>0.1 ± 0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Rauscher cells*</td>
<td>0</td>
<td>166 ± 10</td>
<td>9 ± 1</td>
<td>120 ± 15</td>
</tr>
</tbody>
</table>

*Irradiated Rauscher cells were added at a concentration of 3 × 10^5 cells/mL.
Table 4. Effect of Irradiated Rauscher Erythroleukemia Cells on GM Colony and Erythroid Burst Growth From Blast Cell Colony (bcc)-Derived Progenitors*

<table>
<thead>
<tr>
<th>Stimulus Added</th>
<th>colonies/500 bcc-Derived Cells</th>
<th>GM Colonies</th>
<th>Erythroid Colonies</th>
<th>Mixed Stimulation Bursts</th>
<th>Erythroid Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PHA-LBRM 33 + Ep (1.5 U/mL)</td>
<td>9</td>
<td>171</td>
<td>2</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Irradiated Rauscher cells†</td>
<td>0</td>
<td>159</td>
<td>0</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>Irradiated Rauscher cells + Ep</td>
<td>15</td>
<td>143</td>
<td>13</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

*A representative experiment is shown. Similar results have been obtained in two other experiments.
† Irradiated Rauscher cells were added at a concentration of 2 x 10^3 cells/mL.

was probed with a genomic M-CSF probe (Fig 4). The sizes of these bands ranged from 1.5 to 4.5 kb, which correspond to those expected for M-CSF messages. No signal for IL-3 was detected in poly A-selected and total RNA preparations from Rauscher cells or in total RNA preparations from irradiated Rauscher cells (Fig 4). Positive control for IL-3 expression was represented by total RNA extracted from LBRM 33 cells stimulated for 24 hours with PHA. Negative control was represented by RNA prepared from NIH 3T3 cells. The blot has been further probed for the expression of beta-actin as a control that comparable amounts of RNA were loaded for all the samples investigated (Fig 4).

The blots were also probed for the presence of GM-CSF, G-CSF, and IL-1 transcripts. No hybridization signals for these growth factors were observed (data not shown).

The possible presence of IL-6 in the supernatant of Rauscher cells was tested by bioassay. The supernatant failed to induce proliferation of the murine B cell hybridoma line, B9, or the plasmacytoma cell line (data not shown), indicating that the concentration of IL-6 in the supernatant was <1 pg/mL.

DISCUSSION

The disease induced by the Rauscher virus complex consists of multiple phases (for review see references 36, 37). The first phase, which consists of polyclonal proliferation of erythroid progenitor cells, is due to a direct effect of a product of the SFFV on these cells. These cells cannot be passaged in vivo or in vitro. If the animal survives this state of the disease, one can later detect leukemic cells that can be passaged in vivo and, ultimately, in vitro. These cells are clonally derived and are thought to be the progeny of a rare cell that has undergone a secondary genetic change.

The Rauscher erythroleukemia cell line we have studied expresses ten times more Ep receptors than normal erythroid progenitor cells, and this could be responsible for its transformed state. However, data presented here indicate that this is not likely, since Ep did not restore the growth of the Rauscher erythroleukemia cells when they were cultured at low cell number in FBS-deprived cultures. The lack of response to Ep was not due to down-modulation of Ep receptors, since no differences were observed in the number or affinity of Ep receptors between Rauscher cells grown in the absence or presence of FBS (data not shown).

The results we have obtained indicate that Rauscher erythroleukemia cells produce a factor that supports their growth in an autocrine fashion. The growth of these cells in suspension culture is cell concentration-dependent, and cocultivation under FBS-deprived conditions of low numbers of Rauscher cells with irradiated Rauscher erythroleukemia cells allows for proliferation of the former. Irradiated Friend erythroleukemia cells, which contain both Friend MuLV and SFFV, can substitute for the irradiated Rauscher erythroleukemia cells but SFFV-free erythroleukemia cells from mice infected with F-MuLV cannot (data not shown). This suggests that the growth-promoting property described here may be unique to SFFV-transformed cells.

Irradiated NIH 3T3 cells (data not shown) were ineffec-
active in promoting growth of the Rauscher erythroleukemia cells as were soluble growth factors such as Ep, IL-1, IL-3, IL-4, IL-6, GM-CSF, M-CSF, or conditioned media that contained IL-3 and other growth factors. Rauscher cell growth also was not promoted by Rauscher cell supernatants, membrane preparations, or cells fixed with glutaraldehyde.

Since irradiated cells still promoted colony growth when separated from target cells by an agar layer, the activity may be associated with a soluble factor(s) that is extremely labile.

Irradiated Rauscher erythroleukemia cells also sustained colony formation by three of four IL-3-dependent cell lines tested (particularly 32D cl 23), GM colony formation, and, in the presence of Ep, formation of erythroid bursts and mixed erythroid colonies from normal bone marrow progenitors as well as enriched progenitor cells cultured at low concentrations. This spectrum of growth-promoting activity is similar to that of IL-3. In fact, in the murine system, IL-3 is the only known growth factor that induces GM colonies, and, with Ep, pure and mixed erythroid bursts in FBS-deprived culture (unpublished results). IL-3-dependent cell lines can grow in the presence of growth factors other than IL-3. For example, the DA-1 cell line proliferates in the presence of GM-CSF and/or Ep (12) FDC-P1 cells proliferate in the presence of GM-CSF, and 32D cl 3 cells can be induced to proliferate by G-CSF. However, the 32D cl 23 cell line is exclusively dependent on the presence of IL-3 for proliferation (Table 3 and unpublished results).

Rauscher virus complex in the supernatant could exert a growth promoting effect on normal bone marrow progenitors, either directly or indirectly, via the infection of accessory cells and their activation to produce IL-6 or other hematopoietic growth factors. Although the original Rauscher erythroleukemia cells were reported to lack infectious virus after 14 to 16 passages in culture, the cells used in this study were shown to express both MuLV and SFFV proteins, release reverse transcriptase, and produce virus capable of causing erythroleukemia in adult NIH Swiss mice (data not shown). However, it is unlikely that the viruses released by this line could mediate its growth-promoting effect, since conditioned medium from these cells, which would contain virus, failed to promote cell growth. Furthermore, neutralizing antisera against the viral gp70 did not affect the growth of normal bone marrow progenitors in the presence of irradiated Rauscher erythroleukemia cells.

It is possible that the different growth-promoting activities displayed by Rauscher erythroleukemia cells could be mediated by different factors. Although the cells did not grow in IL-3 or in IL-3-containing conditioned media, the cells still could produce IL-3 and/or a combination of hematopoietic growth factors. Therefore, expression of hematopoietic growth factors was analyzed by Northern blot analysis with the exception of IL-6, whose expression was investigated by bioassay. IL-3 message was undetectable by Northern analysis (Fig 4) as was message for GM-CSF, G-CSF, and IL-1 (data not presented). M-CSF message was detected (Fig 4). However, M-CSF alone does not sustain colony formation from purified normal progenitors under FBS-deprived culture conditions (33) or proliferation of IL-3-dependent cell lines (Table 3). The presence of M-CSF may explain why the majority of Rauscher cell-induced GM colonies were composed of macrophages. IL-6 was not detected by bioassay.

It is not clear if IL-3 plays any role in the control of murine steady-state hematopoiesis because production of IL-3 has not been detected in vivo in any normal murine hematopoietic tissue or in vitro under conditions that sustain hematopoiesis, such as long-term marrow culture or stromal cell culture. Recently, Zipori and Lee (42) reported that cloned endothelial-adjacencies induced growth of stem cells in vitro in the absence of IL-3, GM-CSF, G-CSF, or IL-4 production. They postulated that stromal cells produce an undefined cytokine that is responsible for sustaining hematopoiesis under such conditions. The growth-promoting activity produced by the Rauscher cells, although similar to IL-3, is not IL-3 and may be related to the activity described by Zipori and Lee (42).

In summary, our data suggest that autocrine production of a unique growth factor that sustains the growth of normal hematopoietic progenitor cells plays a role in the transformation of Rauscher erythroleukemia cells.

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