Enhancement of Human Erythroid Progenitor Cell Growth by Media Conditioned by a Human T-Lymphocyte Line

By Anne W. Hamburger

Recent studies have shown that soluble factors elaborated by human T lymphocytes enhance erythroid burst formation by human peripheral blood null cells. This study demonstrates that media conditioned by a long-term T lymphocyte line augmented the growth of erythroid colonies in vitro in the presence of erythropoietin (Ep). ATCC.CCL 119 (CCRF-CEM) was derived from a patient with ALL of T-lymphoblast origin. Cells from the stocks used in these experiments maintained T-cell characteristics as determined by histochemical and rosetting techniques. Increased numbers of 16-day BFU-E were seen when Ficoll-Hypaque separated peripheral blood leukocytes were cultured in the presence of a 10% (v/v) concentration of CCL 119 conditioned medium (CM). CM increased the number of BFU-E even when Ep or fetal calf serum were not growth limiting. CM also increased the number of late BFU-E observed in cultures of nonadherent bone marrow cells. When peripheral blood mononuclear cells were depleted of E-rosetting cells, only small numbers of BFU-E grew. Addition of 119 CM increased the numbers of BFU-E in E-rosette-depleted cultures. CM from B-cell, macrophage, or other T-cell lines tested did not stimulate BFU-E growth as consistently. These studies indicate that CM obtained from ATCC.CCL 119 cells contained burst-promoting activity, one of the factors required for proliferation of early erythroid progenitors.

Human red cell precursors can be detected by their capacity to form colonies in semisolid culture media. Different patterns of colony formation make it possible to detect both primitive progenitors (BFU-E) and more differentiated precursors (CFU-E). Both CFU-E and BFU-E normally require the glycoprotein hormone erythropoietin (Ep) and fetal calf serum for growth in vitro. Similarly, CFU-E growth in vivo requires Ep. However, BFU-E numbers in vivo do not correlate with endogenous Ep levels. Thus, other humoral substances may regulate the early stages of erythropoiesis. An increasing amount of evidence indicates that survival and growth of BFU-E require substances with burst-promoting activity (BPA). Erythropoietin may be needed only for the later stages of erythroid development.

The addition of certain types of cells and/or conditioned media (CM) enhances the growth of primitive erythroid precursors in vitro. CM from human peripheral blood leukocytes, human embryo kidney cells, T cells, or bone marrow cells increase the number of bursts observed in cultures of human peripheral blood or bone marrow cells. Similarly, the addition of murine spleen cell CM enhances the growth of BFU-E in Ep-containing cultures of mouse bone marrow. Most recently, Metcalf et al. have demonstrated that purified GM-CSF stimulates cell division of early erythroid precursors.

The identity of the cell(s) producing BPA has been a subject of intense study and debate. Wagemaker et al. described a high density mouse bone marrow cell that produced BPA. Peripheral blood T cells, adherent cells, and nonadherent non-T cells have been proposed as BPA-producing cells in man.

Problems of preparing completely pure populations of specific cell types from a heterogenous mixture of hematopoietic cells have made identification of the BPA-producing cells difficult. Since cultured cell lines provide a relatively homogenous population of cells, I investigated the effect of CM from hematopoietic-derived cell lines on erythroid progenitor growth. CM from ATCC.CCL 119 (CCRF-CEM), a line derived from a child with T-cell ALL, secreted a factor that enhanced the growth of human peripheral blood (PB) BFU-E. This study describes the biologic activity of the CM.

MATERIALS AND METHODS

Cell Preparation

Peripheral blood and bone marrow samples were obtained from normal volunteers by appropriate informed consent. Peripheral venous blood was collected in preservative-free heparin (10 U/ml) (Fellows Med. Res. St. Louis, Mo.). Mononuclear cells were obtained by centrifugation on Ficoll-Hypaque (FH) (Pharmacia Fine Chemicals, Piscataway, N.J.) at 400 g for 30 min. The recovered cells were washed twice in Hanks’ balanced salt solution (HBBS) with 10% heat-inactivated fetal calf serum (FCS) (Sterile Systems, Logan, Utah). The viable nucleated cell counts, as determined in a hemocytometer using trypsin blue, were routinely more than 95%. Bone marrow cells were aspirated into preservative-free heparin (10 U/ml) and mixed with an equal volume of 3% dextran-saline and sedimented at room tempera-

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culture for 45 min. The cells in the supernate were collected after centrifugation at 150 g for 10 min and washed twice in HBSS with 10% FCS. The viable nucleated cell counts were routinely more than 95%.

In some experiments, nonadherent cells were also used. Peripheral blood or bone marrow cells, at a concentration of $3 \times 10^6$ cells/ml in RPMI 1640 and 10% FCS, were incubated for a minimum of 2 hr at 37°C in plastic tissue culture flasks (Corning, Corning, N.Y.) using volumes of 1 ml/10 sq cm of flask surface. Following incubation, the flasks were gently inverted and the nonadherent cells removed and washed twice in HBSS and 10% FCS. Only 1% of the remaining nonadherent cells obtained from either bone marrow or peripheral blood were positive for α-naphthyl esterase activity.

**BFU-E Assays**

The methyl cellulose assay described by Gregory and Eaves3 was used. The standard media employed was α-MEM (Flow Labs., Rockville, Md.) which contained 0.9% methyl cellulose (Dow Chemical Co., Midland, Mich.), 30% FCS (Sterile Systems, Logan, Utah), 1% deionized26 bovine serum albumin (Sigma, St. Louis, Mo.) 0.1 mmole α-thioglycerol (Sigma), 50 U/ml penicillin, 50 μg/ml streptomycin (Sigma), and 2 U/ml of erythropoietin (Step III Ep Connaught Labs., Willowdale, Ontario). The batch of Ep used had 0.017 μg of endotoxin/unit of Ep as determined using the limulus amebocyte lysate assay.2 To reduce interexperimental variation, a single batch of FCS and α-MEM was used in all experiments reported here. Conditioned media from various cell lines were added to a final concentration of 10% unless otherwise stated. Controls contained the same concentration of RPMI 1640 media with 10% FCS as experimental cultures containing CM.

All methyl cellulose assays were performed in 35-mm plastic Petri dishes (Falcon, VWR Sci. Baltimore, Md.). Each dish contained $2 \times 10^5$ cells in a volume of 1.1 ml unless otherwise specified. Cultures were incubated at 37°C in an atmosphere of 5% CO₂ in air with high humidity.

**Scoring of Cultures**

 Cultures were examined using a Zeiss inverted phase contrast microscope at 100× and 200×. Bursts were defined as colonies consisting of 3 or more subcolonies of erythroid cells or large single accumulations of more than 500 erythroid cells. Hemoglobinization of colonies was initially verified by staining with benzidine22 directly in the cultures. However, cells were most easily recognized by their orange-red coloration.

Peripheral blood bursts were scored between 14 and 16 days after plating. Bone marrow bursts were scored 16–18 days after plating.

**Preparation of Conditioned Media**

ATCC.CCL 119 (CCRF-CEM) was obtained from the American Type Culture Collection (Rockville, Md.). Cells were cultured in antibiotic-free RPMI 1640 media and 10% FCS in stationary suspension culture in 75 sq cm tissue culture flasks. The cells were mycoplasma free as determined by microbiologic assays and Hoechst staining.24 The media was collected when cells had reached a concentration of $10^6$ cells/ml (2–3 days after splitting). Cultures were centrifuged at 400 g for 10 min and the supernatant collected and filtered through 0.22 μM Nalgene filters (Nalge, Rochester, N.Y.). The cell-free conditioned media was then aliquoted and stored at −20°C for up to 3 mo for further experiments. Conditioned media from other hematopoietic cell lines was prepared in a similar manner. Cell lines tested included MOLT 4,25 RPMI 8402,26 ATCC.CCL 120 (CCRF SB),27 ATCC.CCL 213 (Daudi),28 and DHL-2.29

**Cytologic Evaluation of ATCC.CCL 119 Cells**

Cells were cytocentrifuged and stained with Wright-Giemsa for morphology and for peroxidase, nonspecific esterase, Sudan black, and periodic acid Schiff reactivity.31 Cells were also evaluated for E, EA, and EAC rosetting ability by standard methods.32 The ability of cells to phagocytose Latex particles was evaluated as described.31 In addition, cells were evaluated for the presence of human T-cell antigen in the laboratory of Dr. Jun Minowada33 as previously described.

**T-Cell Depletion Experiments**

T-lymphocyte-depleted subpopulations were obtained from peripheral blood mononuclear cells as follows. Mononuclear cells were combined with sheep red cells treated with 2-aminoethylisothiouronium bromide (AET) (Aldrich, Milwaukee, Wisc.). Rosetted cells were separated from non-rosette-forming cells by centrifugation through a gradient of Ficoll-Hypaque (H-F) described by Wahl et al.34 The non-rosette-forming cells were reincubated with SRBC and the Ficoll separation repeated. Rosetted cells were freed of adherent red blood cells by incubation of the suspension at 20°C for 9 min in 0.17 M NH₄Cl. Cells were immediately centrifuged through 100% FCS to remove RBC ghosts. The T-cell-depleted subpopulations contained about 2% E-rosetting cells. The T-
cell-depleted populations were depleted of adherent cells as described above.

**Bioassay of EP**

CM samples were assayed for erythropoietic activity in the posthypoxic mouse bioassay of Camiscoli and Gordon\(^\text{15}\) in the laboratory of Dr. Albert Gordon.

**Statistics**

The probability of differences between samples was determined with the use of the Student's \(t\) test. Four or five plates were scored per point. The results are expressed as a mean ± 1 SEM.

**RESULTS**

**Effect of CM on Growth of BFU-E**

Conditioned media obtained from log phase cultures of ATCC.CCL 119 cells increased the number of 16-day BFU-Es observed in cultures of human PB mononuclear cells (Fig. 1). CM in the absence of Ep did not stimulate the growth of erythroid or granulocyte-macrophage colonies in PB or BM cultures containing \(2 \times 10^7\) cells/ml. CM had no erythropoietic activity in the exhypoxic polycythemic mouse assay down to a level of 0.05 U Ep/ml.

**Effects of CM in Cultures Containing Varying Concentrations of Ep**

The effect of CM on peripheral blood bursts was investigated at different concentrations of Ep. Varying concentrations of Ep were added to cultures of nonadherent PB cells in the presence or absence of CM. The results (Fig. 2) show that enhancement could be observed not only in cultures containing minimal concentrations of Ep, but also in cultures where a maximal response to Ep had been attained. CM reduced the Ep threshold for burst formation by about tenfold.

**Effect of CM in Cultures Containing Varying Concentrations of FCS**

The ability of CM to reduce the requirement for FCS for growth of PB BFU-E was examined. The results of 1 of 3 experiments are illustrated in Fig. 3. The trend in the other experiments was similar, although the quantitative results were different. Large erythroid colonies in control plates did not appear until

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**Fig. 1.** Effect of CM from CCL 119 cells on growth of BFU-E from human peripheral blood mononuclear cells. Cells from individual subjects were cultured in absence (solid bar) or presence (striped bar) of 10% CM. Results are expressed as mean ± SEM (4 plates).

**Fig. 2.** Growth of BFU-E from \(10^8\) peripheral blood mononuclear cells at varying concentrations of Ep in the presence (open circle) and absence (closed circles) of 10% CM. Results are expressed as mean ± SEM (4 plates).

**Fig. 3.** Growth of BFU-E from \(10^8\) peripheral blood mononuclear cells at varying concentrations of FCS in the presence (open circles) and absence (closed circles) of 10% CM. Results are expressed as Mean ± SEM (4 plates).
the cultures contained 30% FCS. The conditioned media increased the number of erythroid colonies at serum levels above 1%. The number of bursts in cultures containing 10% CM and 10% FCS was not significantly different from that seen in cultures containing 30% FCS and no CM. However, no bursts were observed in the complete absence of serum in the presence of CM.

**Effect of Varying Concentrations of CM**

The effect of varying concentrations of CM on BFU-E growth by PB mononuclear cells in the presence of Ep is shown in Fig. 4. The conditioned media induced a dose-related increase in the number of bursts at a constant concentration of 2 U of Ep per culture. A 100% increase in the number of BFU-E as compared to controls was seen at a concentration of 10% CM. At 30% CM, 10 times as many BFU-E grew as in control cultures. No dose-related inhibitory effect was observed under these conditions.

**Cytologic Characteristics of ATCC CCL 119 Cells**

The stocks of CCL 119 cells used in these experiments was evaluated for T-cell properties. The cells were negative for peroxidase, nonspecific esterase, or Sudan black activity. Eighty percent of the cells had periodic acid Schiff (PAS) positive inclusions in the block form. The cells did not phagocytose latex beads. Forty-three percent of the cells formed rosettes with AET-treated sheep red blood cells. No cells had receptors for the Fc portion of IgG as determined using ox red blood cells coated with IgG. Only 1.2% of the cells had complement receptors as determined using sheep red blood cells coated with IgM and complement. No cells had surface immunoglobulins. Eighty to ninety percent of the cells were positive for human T-cell antigen. Thus, the stocks of cells used in these experiments had maintained their T-cell properties.

**Effects of CM From Other Cell Lines**

The effect of conditioned media prepared from other hematopoietic cell lines on growth of PB BFU-E was determined (Table 1). CM from other T-cell lines tested did not consistently enhance growth of BFU-E. CM from two B-cell lines had no effect on erythroid colony growth. CM from a macrophage-like cell line, derived from a patient with diffuse histiocytic lymphoma, had no effect on BFU-E growth (Table 2).

**Fig. 4. Effect of addition of CM to erythroid colony cultures.** Peripheral blood mononuclear cells were cultured in the presence of increasing concentrations of CM at constant concentrations of Ep (2 U/ml) and FCS (30%). Results are expressed as mean ± SEM (4 plates).
Effect of Depletion of Adherent Cells

PB mononuclear cells, obtained after F-H centrifugation, were separated into plastic adherent and nonadherent fractions as described in the Materials and Methods. The growth of BFU-E from unfractionated and nonadherent fractions in the presence or absence of CM was evaluated. The results are presented in Table 2. In most cases, the number of BFU-E per 10^5 cells (grown in the presence of Ep only) was increased in the nonadherent fractions as compared to unfractionated controls. In some cases, however, there was no difference, or decreased numbers of BFU-E were observed per 10^5 cells. However, the addition of CM increased the number of BFU-E in all cases when added to cultures of unfractionated or nonadherent PB Cells. In most cases, the CM-induced increase was more evident in culture containing nonadherent cells.

In addition, bone marrow cells were separated by adherence to plastic flasks as described. The effect of CM on the growth of BFU-E from cells in the adherent and nonadherent fractions was evaluated. The data presented in Table 3 show that in most instances, addition of CM to Ep-containing cultures had no effect on the number of bursts in cultures of unseparated cells. However, CM, which by itself did not stimulate erythroid growth of bone marrow cells, enhanced the growth of Ep-dependent bursts in cultures of nonadherent cells.

Effect of T-Cell Depletion

Nonadherent F-H-separated PB mononuclear cells were obtained as described. The cells were then depleted of E-rosetting cells. The unfractionated and T-cell-depleted populations were then cultured for

Table 3. Effect of Conditioned Media on Fractionated Populations of Human Bone Marrow Cells

<table>
<thead>
<tr>
<th>Subject</th>
<th>Fraction*</th>
<th>+ CM</th>
<th>− CM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UF</td>
<td>70 ± 15†</td>
<td>82 ± 16</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>112 ± 24</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>UF</td>
<td>Not Done</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>65 ± 7</td>
<td>33 ± 9</td>
</tr>
<tr>
<td>3</td>
<td>UF</td>
<td>130 ± 20</td>
<td>160 ± 18</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>300 ± 25</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>4</td>
<td>UF</td>
<td>130 ± 20</td>
<td>110 ± 15</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>128 ± 30</td>
<td>4 ± 4</td>
</tr>
<tr>
<td>5</td>
<td>UF</td>
<td>60 ± 15</td>
<td>23 ± 9</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>80 ± 7</td>
<td>40 ± 5</td>
</tr>
</tbody>
</table>

Bone marrow cells were separated by adherence to plastic flasks. Unfractionated and nonadherent populations were cultured at 2 x 10^5 cells/culture with 2 U/ml Ep.

*UF, unfractionated; NA, nonadherent.
†Mean ± SEM (4 plates).

PB mononuclear cells were depleted of E-rosetting cells and adherent cells as described in the Materials and Methods. CM was added to both T-cell-depleted and unfractionated populations. Cultures contained 2 x 10^5 cells, 2 U/ml Ep, and 10% CM.

Table 4. Effect of 119 Conditioned Media on T-Cell-Depleted Cultures

<table>
<thead>
<tr>
<th>Subject</th>
<th>T Cells Present</th>
<th>CM Present</th>
<th>BFU-E/10^5 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>0</td>
<td>35 ± 4*</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>71 ± 8</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.8 ± 1.8</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
<td>41 ± 6</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>0</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>2.5 ± 1</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
<td>41 ± 4</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>4 ± 3</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>15 ± 3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>0</td>
<td>50 ± 10</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>85 ± 2</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>5 ± 5</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>27 ± 7</td>
<td></td>
</tr>
</tbody>
</table>

PB mononuclear cells were depleted of E-rosetting cells and adherent cells as described in the Materials and Methods. CM was added to both T-cell-depleted and unfractionated populations. Cultures contained 2 x 10^5 cells, 2 U/ml Ep, and 10% CM.

*Mean ± SEM.

BFU-E in the presence or absence of CM. The results from 4 different subjects are presented in Table 4. The number of BFU-E/10^5 cells was significantly reduced in T-cell-depleted cultures. The addition of CM to T-lymphocyte-depleted cultures increased the number of BFU-E/10^5 cells. However, the addition of CM did not restore BFU-E growth to the level seen in cultures containing T lymphocytes.

DISCUSSION

The data presented in this report demonstrate that a cultured cell line, derived from a patient with T-cell ALL, released soluble substances that enhanced growth of BFU-E from cultures of human peripheral blood and bone marrow cells. CM from a variety of cells,9-13,17,36 have been reported to augment the in vitro growth of human or murine erythroid cells. The active component in such media has been variously termed BPA or BFA and has been proposed as the major regulator of early proliferation in the erythroid pathway. BPA-like factors have also been isolated from human urine37 and serum.38 However, the relationship of these factors to one another or to the factor described here is not known.

In this study, CM reduced the threshold amount of Ep required for erythroid burst formation in culture. In addition, the dose-response curve was shifted to the left, and the maximum number of BFU-E increased twofold. This, and the data showing increased numbers of bursts with increasing amounts of CM, indicated that the number of BFU-E and their sensitivity to EP was related to the concentration of the
CM. This effect is similar to the action of BPA reported by Porter,11 Iscove,7 and Aye9 and would support the theory that CM may promote growth and maturation of early erythroid stem cells.39 CM from ATCC.CCL 119 cells also reduced the threshold of erythroid growth supporting substances in the batches of FCS used in these experiments. We are currently testing the effect of CM in erythroid burst cultures containing no FCS.40

The in vivo cellular source of BPA is not yet known. It has been proposed that bone marrow macrophages,9 peripheral blood T lymphocytes,10 or peripheral blood nonadherent non-T mononuclear cells18 produce factors with BPA-like activity. It had been hoped that the use of a cell line that had retained T-cell markers would clarify this point. ATCC.CCL 119 has retained many of its T-cell characteristics as assessed by antigenic, histochemical, and functional tests. The fact that a T-cell line secreted BPA lends support to the theory that T cells play a regulatory role in erythropoiesis.

However, T cells may not be the cells solely responsible for the production of BPA. Adherent cells have been shown to produce BPA found in leukocyte-conditioned media.9 It is possible that T cells, in cooperation with a second cell population, modulate the growth of BFU-E. For example, the effect of the 119 CM was observed only when populations of bone marrow nonadherent cells were assayed. The effect on PB bursts was more marked when nonadherent cells were used. The addition of CM to cultures containing cells capable of producing their own BPA may have exceeded the optimal concentration required for activity. However, when adherent cells were present at very limiting concentrations (as they probably were in the plastic “nonadherent” populations), the addition of 119 CM may have increased macrophage production of BPA.

The present observations provide evidence that a T-lymphocyte line, passaged for 15 yr, produced a humoral factor that may be important in the regulation of human erythropoiesis. Such a cell line can provide a reliable source of BPA for purification studies. The use of a purified molecule, rather than crude conditioned media, should lead to a better understanding of the mode of action of BPA and regulatory interactions in the erythropoietic pathway.

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