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Production of erythroid-potentiating activity by a human T-lymphoblast cell line

(erythroid colonies/lymphokine/burst-promoting activity/T lymphocytes/colony-stimulating factor)

DAVID W. GOLDE, NOELLE BERSCH, SHIRLEY G. QUAN, AND ALDONS J. LUSIS

Division of Hematology-Oncology, Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024

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ABSTRACT **We derived a human T-lymphoblast cell line (Mo) that constitutively elaborates certain lymphokines. The Mo cells produce a colony-stimulating factor necessary for the growth of human granulocyte–monocyte precursors *in vitro* as well as an erythroid-potentiating activity (EPA) that enhances the proliferation of human erythroid progenitors *in vitro*. In the presence of serum, the EPA in Mo-conditioned medium stimulated the growth of small and large erythroid colonies almost 2-fold. EPA was also produced in serum-free medium, and, when assayed in serum-free cultures of human erythroid progenitors, it stimulated colony growth about 3-fold. The EPA produced by the Mo cell line did not stimulate normal murine erythroid progenitors (CFU-E) or Friend erythroleukemia cell growth *in vitro*. EPA was inactivated by protease treatment but was remarkably heat stable, with most of the activity recovered after boiling for 15 min. Preliminary biochemical characterization suggests that EPA is an acidic glycoprotein with molecular weight approximately 45,000. EPA is clearly separable from colony-stimulating factor on the basis of heat stability and gel-filtration chromatography. The present observations provide strong support for the concept that activated T cells produce humoral factors important in the regulation of erythropoiesis. The availability of a cell line producing human EPA should facilitate the characterization of the protein and permit definitive studies of its biologic effects.**

It has long been recognized that erythropoietin is the primary humoral stimulator of erythropoiesis. Recent studies, however, have led to the definition of another physiologic modulator important in regulating the early steps in erythroid development (1, 2). In 1976 Aye (3) demonstrated that conditioned medium from peripheral blood leukocytes enhanced the growth of erythroid colonies *in vitro*. The activity in leukocyte-conditioned medium that augments erythroid proliferation *in vitro* has been referred to as burst-feeder activity (4), burst-promoting activity (5), and erythroid-enhancing activity (6). Although stimulators of *in vitro* erythropoiesis have been obtained from embryonic fibroblasts (7), leukocyte- and bone-marrow-conditioned media (3, 4, 8), and urine from anemic patients (9), the most common source for the production of these modulators has been lectin-stimulated lymphoid populations. Thus, phytohemagglutinin-, concanavalin A-, and pokeweed mitogen-stimulated lymphocytes have been shown to release factors that stimulate erythropoiesis *in vitro* (1, 5, 10, 11). Many of the cell types that produce erythroid-potentiating activity (EPA) also produce the colony-stimulating factor (CSF) necessary for the growth of granulocyte–monocyte colonies *in vitro* (3, 6, 12).

We have derived a human T-lymphocyte cell line (Mo) that produces a potent CSF for human granulocyte–monocyte progenitor cells (12). We now report that the Mo cell line produces a separable protein that stimulates the growth of human erythroid progenitors *in vitro*.

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MATERIALS AND METHODS

Mo Cell Line. The Mo cell line was established with spleen cells from a patient with a T-cell variant of hairy-cell leukemia (13). These cells have been growing in continuous culture for over 2 years, and they maintain the properties of T lymphoblasts. More than 60% of the cells rosette with sheep erythrocytes, and they carry the tartrate-resistant isozyme 5 of acid phosphatase characteristic of hairy-cell leukemia. There is no evidence for immunoglobulin synthesis by these cells (14). The Mo cells are not infected with Epstein–Barr virus and they are lysed by antithymocyte globulin in the presence of complement. The cells respond to phytohemagglutinin by increased incorporation of [^{3}H]thymidine (14). We have previously reported CSF production by these cells (12) and the elaboration of a molecule that inhibits the migration of neutrophils (15).

Growth of Erythroid Progenitors *In Vitro*. Small erythroid colonies (CFU-E) and large erythroid clones (BFU-E) were grown in methylcellulose by using normal human bone marrow and peripheral blood (16, 17). The basic methylcellulose technique was adapted from the method of Iscove *et al.* (18), with 0.8% methylcellulose (Dow), alpha medium (Flow Laboratories) with 30% fetal calf serum, 0.1 mM α-thioglycerol (Calbiochem), and penicillin and streptomycin. Human bone marrow CFU-E were counted at 7–8 days, and clusters of eight or more hemoglobinized cells were enumerated. Human peripheral blood and bone marrow bursts were counted at 14 days as erythroid clusters of 50 cells or more. Human urinary erythropoietin was obtained from the Division of Blood Diseases and Resources, National Heart, Lung, and Blood Institute. The lot used had an activity of approximately 44 units per mg of protein.

A serum-free erythroid culture technique was used to assay EPA produced by the Mo cells in a serum-free environment. The serum-free methylcellulose culture technique for human CFU-E and BFU-E was modified from the method of Guilbert and Iscove (19). The culture medium contained 1% bovine serum albumin (Sigma), transferrin (350 μg/ml), ferric chloride (1.6 μM), sodium selenite (0.1 μM), insulin (1 ng/ml) (20), and human growth hormone (200 ng/ml) (16). Using this formula, we could grow both CFU-E and peripheral blood bursts in the absence of serum components other than albumin. Murine CFU-E cultures were prepared as described (17). Friend erythroleukemia cells (GM-86, clone 745) were cloned in serum-free methylcellulose culture with alpha medium containing 0.5% bovine serum albumin, and colonies of eight or more cells were enumerated at 72 hr (20).

CSF was assayed by the agar technique previously described (12, 21). The target cells were light-density nonadherent bone marrow cells (separated on Ficoll/Hypaque) obtained from normal volunteers.

Abbreviations: BFU-E, burst-forming unit (erythroid); CFU-E, colony-forming unit (erythroid); CSF, colony-stimulating factor; EPA, erythroid-potentiating activity.

RESULTS

The Mo cells continuously elaborated a factor that potentiated the growth of human erythroid colonies *in vitro*. This activity was prominently discernible in cultures of human bone marrow to which no exogenous erythropoietin had been added. In the methylcellulose system containing 30% fetal calf serum (selected lot), we normally obtained 25–100 CFU-E per 10^5 normal human bone marrow cells in plates that did not contain added erythropoietin. The addition of Mo-conditioned medium (50 μl) to these cultures caused an increase in CFU-E of 141% ± 6% in 13 separate experiments. Dose–response curves were constructed for Mo-conditioned medium by using a suboptimal concentration of erythropoietin (0.1 unit/ml). In the serum-containing system, activity could regularly be seen at 10 μl/ml of Mo-conditioned medium and maximal activity was seen at 50 μl/ml (Fig. 1 *Upper*). When conditioned medium from Mo cells grown in liquid suspension with serum-free medium was used and this was added to serum-free cultures of human CFU-E, prominent potentiation of erythroid cloning was observable (Fig. 1 *Lower*). At optimal concentrations of Mo-conditioned medium (50 μl/ml), the growth of CFU-E in serum-free methylcellulose plates approached that of the serum control. At erythropoietin concentrations of 1 unit/ml and greater, potentiation of CFU-E by high concentrations of Mo-conditioned medium was frequently not observable. This phenomenon was due to the presence of interacting inhibitors which could be removed by boiling the conditioned medium for short periods of time (Fig. 2). The boiled conditioned medium gave prominent potentiation at all concentrations of erythropoietin tested up to 2 units/ml (Fig. 2).

The marked heat stability of the EPA provided a means for separating it quickly from the CSF also produced by the cell line. Fig. 3 shows a comparison of the heat-stability curves for CSF and EPA. The conditioned medium was immersed in boiling water for varying periods of time and then assayed for CSF and for EPA. After 30 min of boiling, there was a loss of 95% of the CSF activity while most of the EPA remained.

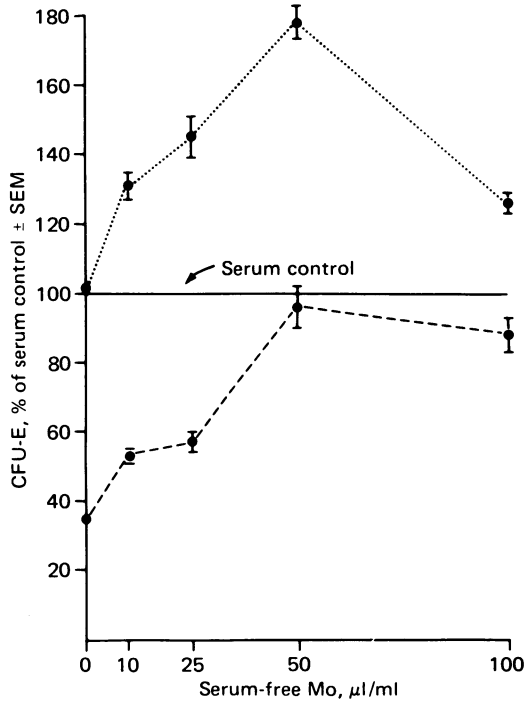

FIG. 1. Human bone-marrow-derived CFU-E response to Mo-conditioned medium in standard serum-containing culture (30% fetal calf serum) (*Upper*) and in a serum-free system (*Lower*). Cultures contained 0.1 unit of erythropoietin per ml.

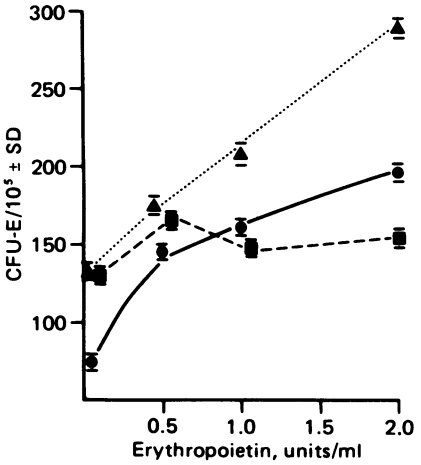

FIG. 2. Relationship between erythropoietin concentration and response to 50 μl of Mo-conditioned medium per ml. Boiling of Mo-conditioned medium for 10 min removed inhibiting activity seen at erythropoietin concentrations of 1 unit/ml or greater. ●, Control; ■, Mo; ▲, Mo boiled.

Human peripheral blood burst formation was augmented by boiled Mo-conditioned medium at all concentrations of erythropoietin tested up to 2 units/ml (Fig. 4). Conditioned medium from Mo cells grown serum free was an equally good stimulator of both BFU-E and CFU-E. Table 1 shows the response of boiled, serum-free, Mo-conditioned medium assayed in serum-free cultures of human peripheral blood BFU-E. BFU-E in the serum-free system were about 20% of that observed in the serum-containing system. The serum-free, Mo-conditioned medium stimulated BFU-E at all levels of erythropoietin tested up to 2 units/ml.

No significant augmentation in erythroid colony formation was noted when Mo-conditioned medium was added to cultures of murine bone marrow CFU-E (data not shown). Also, the Mo-conditioned medium had no effect on the cloning of Friend erythroleukemia cells. The Mo-conditioned medium did not contain bioassayable erythropoietin, as tested in the exhypoxic mouse assay (kindly performed by Peter Dukes, Childrens Hospital of Los Angeles).

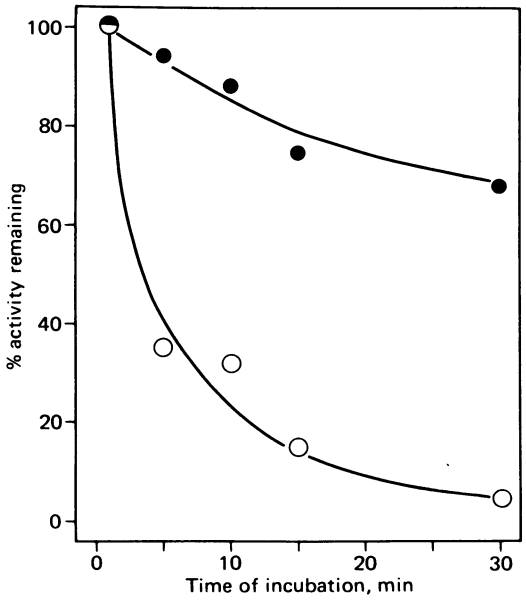

FIG. 3. Heat-stability curves for CSF (○) and EPA (●) present in Mo-conditioned medium.

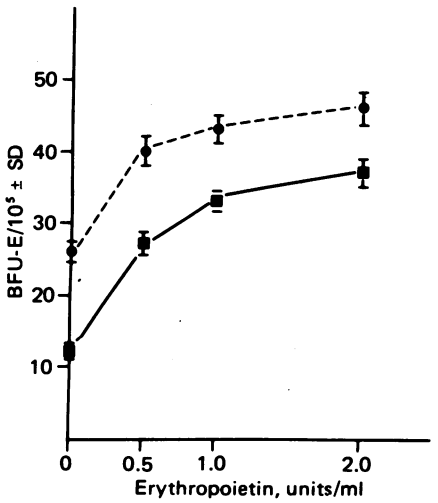

FIG. 4. Effect of boiled Mo-conditioned medium on peripheral blood BFU-E. ■, Control; ●, Mo boiled.

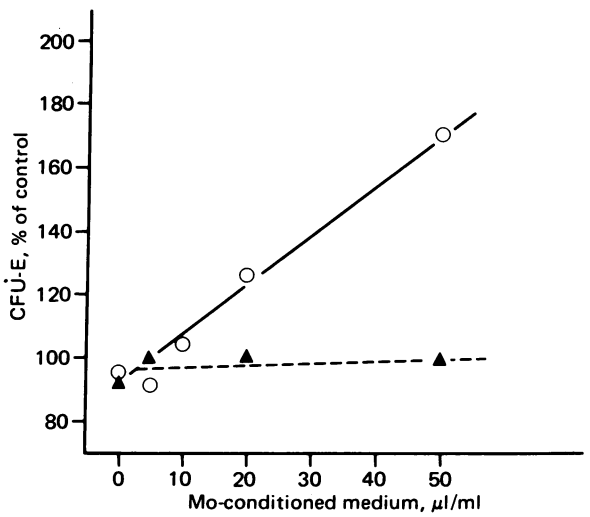

FIG. 5. Protease inactivation of EPA. Mo-conditioned medium was incubated for 1 hr at 37°C with 0.3 mg of Pronase per ml (Calbiochem) (▲) or a buffer control (○) and then boiled for 5 min to destroy Pronase activity. Heat-inactivated Pronase had no effect on CFU-E colony formation.

Preliminary biochemical characterization of EPA was undertaken to determine the chemical nature and possible heterogeneity of the activity. The Mo EPA appears to be a protein because the activity was destroyed by protease treatment (Fig. 5). When subjected to gel filtration, the EPA eluted as a single major peak, corresponding to an apparent molecular weight of about 45,000, with a higher molecular weight shoulder (Fig. 6). The shoulder could have resulted from nonspecific aggregation with serum albumin, which accounted for the bulk of the protein in serum-containing Mo-conditioned medium. The EPA was separable by gel filtration from the T lymphocyte CSF, which eluted in a volume corresponding to a molecular weight of 34,000 (Fig. 6). All of the EPA activity in Mo-conditioned medium was bound by DEAE-Sephadex at pH 7.4; by use of a linear NaCl gradient, it was eluted from the resin as a single broad peak between 0.15 and 0.25 M NaCl (Fig. 7). When subjected to preparative isoelectric focusing in granulated gel, the bulk of the EPA was found in the very acid portions of the gel corresponding to pI 3.5–4.8 (data not shown). The EPA was bound by concanavalin A-Sepharose, and most of the activity could be recovered from the lectin by elution with 0.2 M methyl-α-D-mannoside (data not shown). These findings suggest that EPA is an acidic glycoprotein of molecular weight about 45,000.

DISCUSSION

T lymphocytes have important interactions in the regulation of hematopoiesis (22). Lectin- and antigen-stimulated T lymphocytes produce a CSF (23), and stimulated T cells produce humoral mediators that augment early erythroid colony formation *in vitro* and stimulate the formation of mixed colonies (10, 11, 22). Mixed colonies of human hematopoietic cells have been grown *in vitro*, and their formation is dependent upon factors elaborated by mitogen-stimulated lymphocytes (11). Similarly, human leukemia cells require the presence of a T-lymphocyte factor for the formation of large colonies *in vitro* (24). Unstimulated T lymphocytes also augment human burst formation *in vitro* (25) and stimulate the murine spleen colony-forming unit (26).

We derived a human T-lymphoblast cell line from a patient with a T-cell variant of hairy-cell leukemia (13). This T-cell line is unique because the cells liberate many of the products known to be elaborated by lectin- or antigen-stimulated normal T cells. We have previously shown that the Mo cells elaborate a potent CSF and a mediator (neutrophil-inhibitory factor) that inhibits the migration of mature neutrophils *in vitro* (12, 15). There also is evidence that they produce a factor that stimulates human acute myelogenous leukemia cells (24, 27). In addition, we have preliminary evidence that the Mo-conditioned medium stimulates human mixed colonies *in vitro*.

Table 1. Effect of EPA on burst formation *in vitro* (BFU-E)

Erythropoietin, units/ml	Serum-free Mo-conditioned medium (50 μl/ml)	Serum-free BFU-E × 10^{-5}
—	−	2
—	+	9
0.5	−	3
0.5	+	11
1.0	−	5
1.0	+	13
2.0	−	6
2.0	+	11

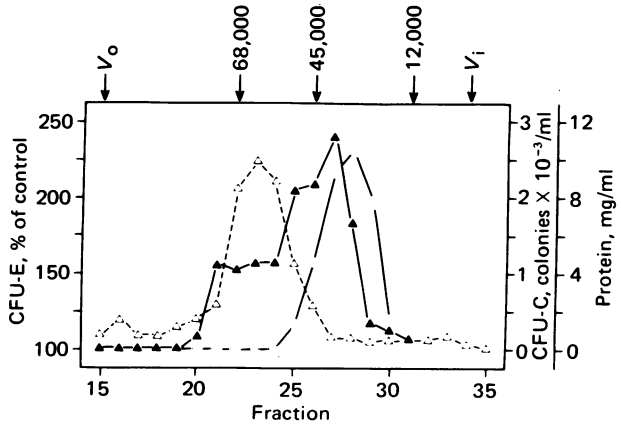

FIG. 6. Gel filtration of Mo-conditioned medium. Medium was concentrated 10-fold by lyophilization; 4 ml of the concentrate was chromatographed on a 2.6 × 55 cm Ultrogel AcA-44 (LKB) column equilibrated with 20 mM sodium phosphate/0.15 M NaCl, pH 7.4. The column was calibrated with blue dextran (void volume, V_o), bovine serum albumin (M_r = 68,000), ovalbumin (M_r = 45,000), cytochrome *c* (M_r = 12,000), and phenol red (included volume, V_i). The recovery of both EPA and CSF activity was greater than 90%. △, Protein concentration; ▲, EPA activity; ○, CSF activity.

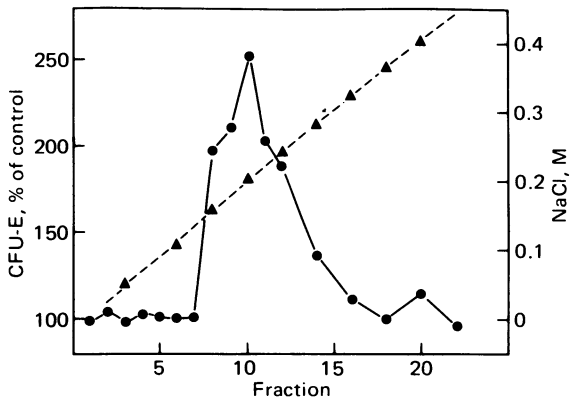

FIG. 7. DEAE-Sephadex chromatography of EPA. Mo-conditioned medium was dialyzed against 20 mM Tris·HCl (pH 7.4), and 15 ml of this was applied to a 1 × 15 cm column of DEAE-Sephadex equilibrated with the same buffer. The column was then developed with a linear salt gradient, from 0 to 0.5 M NaCl in equilibration buffer (total volume, 200 ml). Salt concentration (▲) was determined by conductivity. The recovery of EPA activity (●) was about 120%. The T lymphocyte CSF eluted in a relatively sharp peak between 0.18 and 0.22 M NaCl.

It is now clear that although erythropoietin is important in the latter stages of erythroid development, the earliest erythroid progenitors are stimulated by a material referred to as burst-promoting activity, burst-feeder activity, or erythroid-potentiating activity (EPA). The existence of a modulator of the early events in erythropoiesis was predicted on the basis of studies showing a lack of responsiveness of BFU-E to erythropoietin *in vivo* (28). Recently, burst-promoting activity has been measured in human serum (29).

In this report we have shown that the Mo T-lymphocyte cell line produces a factor that enhances human erythroid cell growth *in vitro*. The factor, termed EPA, appears to be an acidic glycoprotein with molecular weight about 45,000. The EPA is clearly separable from CSF on the basis of its remarkable heat stability, and the two factors can also be resolved by gel-filtration chromatography. The EPA produced by the Mo line does not appreciably augment the product of erythroid colonies (CFU-E) from mouse bone marrow. The absence of an effect on murine CFU-E is evidence against the concept that EPA is erythropoietin.

EPA is elaborated by the Mo cells into a serum-free medium and may be assayed in a serum-free system that supports the growth of human CFU-E and BFU-E. In this system the addition of Mo-conditioned medium at optimal concentrations permits cloning in the serum-free system with efficiency approaching that obtained in the standard system containing 30% fetal calf serum.

The present observations provide evidence that activated human T lymphocytes produce a humoral factor that may be important in the regulation of human erythropoiesis. The availability of a human cell line producing EPA should facilitate the characterization and purification of the protein and allow for conclusive studies of its physiologic role.

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