We report that erythroid-potentiating activity (EPA), known to stimulate the proliferation of normal human erythroid precursors in vitro, has a growth-promoting effect on human K562 erythroleukemia cells and Friend mouse erythroleukemia cells. Detailed studies were carried out using an EPA produced by a human T-lymphoblast line (Mo). Although EPA has not been purified to homogeneity, several observations indicate that the factor elaborated by Mo cells that stimulates erythroleukemia cell growth is the EPA molecule. The erythroleukemia growth factor cofractionates with EPA using gel exclusion chromatography, isoelectric focusing, and ion exchange chromatography. In addition, the activities exhibit similar kinetics of heat inactivation. A granulocyte-macrophage colony-stimulating factor also elaborated by Mo cells had no effect on the growth of the erythroleukemia cells. Other sources of EPA, such as peripheral blood leukocyte-conditioned medium, preparations from urine of anemic patients, and medium conditioned by a human monocytelike cell line, stimulated erythroleukemia cell growth. Mouse sources of EPA (termed "burst-promoting activity") stimulated mouse but not human erythroleukemia cells. The availability of cell lines apparently responsive to EPA should prove useful for examining the mode of action of this regulator of erythropoiesis.

**MATERIALS AND METHODS**

**Mo Cell Line**

The Mo cell line was established from spleen cells of a patient with a T-cell type of hairy cell leukemia. The cells have the characteristics of T lymphoblasts and carry a marker for hairy cell leukemia, the tartrate-resistant isozyme 5 of acid phosphatase. The Mo cells constitutively elaborate a granulocyte-macrophage colony-stimulating factor (CSF) that is physically separable from EPA, as well as other T-cell lymphokines.

**Leukemia Cell Lines**

We obtained the K562 cell line from Dr. John Fahey at UCLA. This cell line was originally established by Lozzio and Lozzio from a patient with chronic myelogenous leukemia. Recently, it has been shown that many of the available strains of K562 cells have erythroidic membrane markers and are able to synthesize hemoglobin when induced to differentiate. These cells constitute the only available human erythroleukemia cell line and are analogous in many ways to the Friend-virus-infected mouse erythroleukemia cells. The K562 cells carried in our laboratory have the characteristics of erythroid progenitors and are karyotypically similar, if not identical, to the original cell line. Mouse Friend-virus-infected erythroleukemia cells (clone 745) were a gift from Dr. Charlotte Friend, New York. These cells may be induced by dimethyl sulfoxide to undergo erythroid differentiation and hemoglobin synthesis. The human and the mouse erythroleukemia cell lines were maintained in continuous suspension culture in alpha medium supplemented with 5% fetal bovine serum (Gibco, Grand Island, N.Y.) and antibiotics.

**Assay for Erythroleukemia Cell Colony Formation**

The methylcellulose technique was used to clone K562 and Friend erythroleukemia cells in vitro in a system similar to that described for normal erythroid progenitor cells. A serum-substituted modification was used in which fetal bovine serum was replaced by 0.5% bovine serum albumin. The K562 or Friend erythroleukemia suspension cultures were fed 1-3 days before cloning. Together with...
the material to be tested, 3 x 10⁴ erythroleukemia cells were plated per culture dish containing alpha medium (Gibco), 0.5% bovine serum albumin (Sigma Chemicals, St. Louis, Mo.), 0.8% methylocel- lulose (Dow Laboratories), 0.1 mM α-thioglycerol (Calbiochem), and antibiotics (Gibco) in a total volume of 1 ml. Duplicate or triplicate plates were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C. After 4-5 day incubation, clusters of 8 or more cells were counted using an inverted microscope. The day 4 mean cloning efficiency was relatively constant at 25% (15%-35%) for K562 and at 30% (20%-40%) for Friend erythroleukemia cells. In our hands, replicate plates did not vary by more than 4%.

**Assays for Normal Hematopoietic Cells**

Erythroid colonies (CFU-E) and bursts (BFU-E) were cultured in methylcellulose using normal human bone marrow and peripheral blood, respectively, as previously described. Human bone marrow CFU-E were counted after 7-8 day incubation as red-brownish clusters of 8 or more hemoglobin-containing cells. Bursts from peripheral blood with 50 or more hemoglobin-containing cells were counted 14 days after plating using an inverted microscope at 100× magnification. Colony-stimulating factor was assayed by the agar culture technique. Ficoll-Hypaque-separated light density nonadherent human bone marrow cells were plated and colonies enumerated after 11-14 days.

**Gel Filtration Chromatography**

Mo cell conditioned medium was concentrated tenfold by lyophilization. Four milliliters (approximately 150 mg protein/ml) was chromatographed on 2.6 x 55 cm Ultrogel AcA 44 (Pharmacia, Piscataway, N.J.) columns equilibrated and eluted with 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.4. Sixty fractions of 6 ml each were collected. Molecular weight markers were blue dextran (void volume), bovine serum albumin (68,000), ovalbumin (45,000), cytochrome-C (32,000), and 4-methylumbelliferone (- included volume). Fractions were assayed for EPA, using the BFU-E or CFU-E assay, and for stimulation of K562 plating efficiency. The samples were stable for at least 4 mo when stored at -20°C. Protein was determined using a dye binding procedure with reagents purchased from BioRad Laboratories. Bovine serum albumin was used as the standard.

**Ion Exchange Chromatography**

Ion exchange chromatography was performed as previously described. Mo cell-conditioned medium was dialyzed against 20 mM Tris-HCl (pH 7.4), and 15 ml was applied to a 1 x 15 cm DEAE Sephadex column equilibrated with the same buffer. The column was developed with a linear salt gradient (from 0 to 0.5 M NaCl) and 30 fractions of 6 ml each were collected.

**Isoelectric Focusing**

Flat bed isoelectric focusing was performed on an LKB multiphor apparatus. Mo cell conditioned medium was incorporated into a 100-ml total volume slurry of 5 g Ultrodex granulated gel (LKB) together with 1% pH 4-6 ampholines and 1% pH 3.5-10 ampholines (LKB). This mixture was poured on the flat bed and was focused at 8 W constant power for 16 hr at 5°C. The gel was then subdivided into 32 fractions and extracted with 10 ml 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.4, dialyzed against phosphate-buffered saline, and assayed for ability to stimulate K562 plating efficiency.

**RESULTS**

In a previous report we described that when Mo cell conditioned medium was fractionated by gel filtration chromatography, the EPA activity was eluted in a major peak corresponding to a molecular weight of about 45,000 daltons with a higher molecular weight shoulder. Erythroid-potentiating activity eluted differently from the CSF, which was mainly found in a volume corresponding to a molecular weight of approximately 34,000.

We repeated these experiments in an identical way as described. After gel filtration, fractions 34-44 containing EPA and fractions 45-54 with CSF were separately pooled and tested for stimulation of K562 plating efficiency and on normal human erythroid and granulocytic precursors. Fractions with EPA activity were found to prominently stimulate the plating efficiency of K562 cells, whereas the CSF-containing material had no effect on K562 cells. A dose-response curve for EPA stimulation of K562 plating efficiency is shown in Fig. 1. In contrast to the stimulation observed with preparations of EPA obtained from pooled gel filtration fractions, most batches of crude Mo cell conditioned medium tested gave little or no stimulation of K562 plating efficiency (data not shown). The inactivity of the crude conditioned medium likely relates to the presence of inhibitors. Heat-labile factors inhibiting the formation of colonies from normal erythroid progenitors are present in crude

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**Fig. 1.** Effect of EPA obtained by gel filtration chromatography on the stimulation of K562 plating efficiency and BFU-E. Gel filtration of Mo cell conditioned medium was performed as described. Fractions with EPA (nos. 34-44) were pooled for these studies. At higher concentrations of EPA than shown here, inhibition of K562 plating efficiency and BFU-E was observed. A representative experiment is shown. In the control plates of the demonstrated experiment the absolute colony count was 832/3000 for K562 cells, and 300/3 x 10⁴ cells plated for BFU-E.
Mo cell conditioned medium, and the decrease in K562 plating efficiency at high concentrations of EPA suggests that inhibitors may not have been completely removed by gel exclusion chromatography. In addition, normal erythroid progenitors appear to be more sensitive than K562 cells to EPA (Fig. 1). Concentration and purification of EPA is necessary to detect a stimulatory effect on K562 plating efficiency.

A series of studies were performed to demonstrate that the K562 stimulating activity in Mo cell conditioned medium was EPA. To determine the molecular weight of the factor stimulating the K562 plating efficiency, gel filtration chromatography was performed and fractions were assayed on K562 cells and on normal erythroid precursors (Fig. 2). In repeated chromatographic procedures, the elution profile for EPA assayed on CFU-E or BFU-E (data not shown) was similar to the elution pattern for the proliferation factor of K562 cells; both factors have an apparent molecular weight of about 45,000. The CSF for granulocytes and macrophages, as mentioned above, eluted differently from EPA as a single peak corresponding to a molecular weight of approximately 34,000.5,23

The K562 growth factor in Mo cell conditioned medium also resembled EPA in terms of behavior on ion exchange chromatography and isoelectric focusing (Fig. 3). Previous studies have shown that the EPA binds to DEAE-Sephadex at pH 7.4, and it is eluted with a linear NaCl gradient as a single broad peak between 0.15 and 0.25 M NaCl.3 When subjected to isoelectric focusing, EPA focused at about pH 3.5–4.8 with a smaller peak at about pH 6.0.11 As shown in Fig. 3B, the activity stimulating K562 cells is similar to EPA in these characteristics. The K562 growth factor also focused with a broad heterogeneous peak between pH 3.5 and 4.8 and with a second smaller peak at pH 6.0. Erythroid-potentiating activity assayed on K562 cells was also found to bind to concanavalin-A-Sepharose, and the activity could be

![Fig. 2. Gel filtration chromatography of Mo cell conditioned medium. Serum containing Mo cell conditioned medium was concentrated and chromatographed on Ultrogel as described. Fractions of 6 ml each were collected, and 50 μl of each was assayed for stimulation of K562 plating efficiency. The data obtained in repeated experiments were found to be superimposable on results from earlier experiments where CFU-E was used for assaying EPA. They are shown here for comparison. For K562, the absolute number of colonies was 504 in the control plates (16.8% plating efficiency).](image-url)

![Fig. 3 (A) Ion exchange chromatography of Mo cell conditioned medium was performed as described. The column was developed with a salt gradient as indicated. Thirty fractions of 6 ml each were collected. Fifty microliters of the fractions were added to the 1 ml K562 culture plates and assayed for stimulation of K562 plating efficiency. Shown is a representative experiment. The untreated K562 control cultures contained 536 colonies. (B) Flat bed isoelectric focusing of Mo cell conditioned medium was performed as described. After extraction of EPA from the gel, 50 μl of each fraction was assayed for stimulation of K562 plating efficiency. The results of a representative experiment performed in duplicate are shown. The control culture plates contained 720 K562 colonies.](image-url)
eluted from the lectin with 0.2 M methyl-α-mannoside (data not shown).

As reported earlier, EPA possesses remarkable heat stability, which provided a useful means for quickly separating it from the more heat-labile CSF. To complete the arguments indicating that the K562 cells respond to EPA in the Mo cell conditioned medium, the heat stability properties were compared. Material stimulating the plating efficiency of K562 cells, obtained by pooling EPA-containing fractions after gel filtration as described, was immersed in a boiling water bath for varying periods of time and assayed. Figure 4 shows that EPA preserved most of the activity stimulating K562 after boiling for 30 min.

Mouse Friend erythroleukemia cells were examined for response to crude Mo cell conditioned medium and to EPA obtained by gel filtration. Like K562 cells, the mouse erythroleukemia cells did not respond to crude Mo cell conditioned medium (data not shown), but preparations of EPA obtained by gel filtration caused a dose-dependent increase of Friend erythroleukemia cell colony formation with an augmentation as high as 148% of control (Fig. 5).

Other human and mouse sources known to contain growth-promoting activity for normal erythroid progenitors were tested for activity on both human K562 erythroleukemia cells and mouse Friend leukemia cells. The results are demonstrated in Fig. 5. Conditioned medium from phytohemagglutinin-stimulated peripheral human blood leukocytes that contains "erythroid-enhancing activity" was prepared and found to augment the proliferation of both human and mouse erythroleukemia cells. A preparation of "regulatory protein" (RP) with burst-promoting activity present in the urine of anemic patients (gift of Dr. Peter Dukes) also stimulated K562 plating efficiency up to 131% of control. We further tested conditioned medium from the human monocyte-like cell line, GCT (provided by Dr. Camille Abboud, Rochester) reported to produce a factor that enhances normal human erythroid colony formation for a stimulatory effect on K562 cells, and found it caused an increase in erythroleukemia cell plating efficiency.
These erythroid-potentiating and -enhancing activities of human origin may also be analogous to burst-promoting factors from mouse sources. Conditioned medium from concanavalin-A-stimulated mouse spleen cells with "burst-promoting activity" was prepared as described1,3 and tested on human K562 and mouse Friend erythroleukemia cells. Only the neoplastic mouse erythroid cells were found to respond to mouse burst-promoting activity, but not the human K562 cells, suggesting that factors with EPA show some order specificity. This conclusion was supported by the finding that a semipurified preparation of "burst-feeder activity"4 (provided by Dr. Gerard Wagemaker, The Netherlands) from conditioned medium of mouse bone marrow was highly active in stimulating Friend erythroleukemia cells, but was not effective on the human K562 cells (Fig. 5).

DISCUSSION

In this report we show that gel filtration fractions containing EPA derived from the Mo cell line stimulate the plating efficiency of human K562 leukemia cells and mouse Friend erythroleukemia cells in vitro. Although homogeneous preparations of EPA were not available, there is strong evidence for the identity of EPA and the activity stimulating erythroleukemia cell plating efficiency. The activities cofractionated using gel filtration chromatography and exhibited similar charge and heat stability. These results are summarized in Table 1. Moreover, other sources of human and mouse EPA (or burst-promoting activity) stimulated erythroleukemia cell growth in a manner that is concordant with the observed order specificity of the factors interacting with normal erythroid progenitors. That is, the human factors stimulated both human and mouse cells, while the mouse factors were active only on mouse cells.

Apparentiy, EPA in the Mo cell conditioned medium is related to factors termed "erythroid-enhancing activity" from conditioned medium of phytohemagglutinin-stimulated peripheral human blood leukocytes and conditioned medium from the GCT cell line. These factors of human origin were active in stimulating cell proliferation of normal human CFU-E and BFU-E.25,27 Furthermore, EPA from the Mo cell line and from the GCT cell line are both acidic glycoproteins that exhibit remarkable heat stability.25 The mouse factors with "burst-promoting activity" elaborated by concanavalin-A-stimulated mouse spleen cells resemble EPA in terms of glycosylation and molecular weight,3 but they were active only on mouse BFU-E and did not influence the proliferation of mouse CFU-E. Our results, showing that human and mouse components with activity related to EPA also stimulate the proliferation of erythroleukemia cells, confirm that these factors may act at an early level of erythroid determination.

Our findings indicate that K562 cells may provide a useful assay system in the purification of EPA, since the clonogenic assay of K562 in methylcellulose is a sensitive, reproducible, and reliable method for measuring small amounts of partially purified EPA. The K562 cells have also been reported to respond to erythropoietin.28 The responsiveness of K562 cells to EPA of different sources suggests that these cells retain the postulated receptor for EPA characteristic of normal erythroid progenitors.3 In providing a homogeneous population of cells responsive to EPA, the K562 human erythroleukemia cells may be useful for molecular studies on the action of EPA. The use of such a cell line avoids possible artifacts from interactions with other cell populations present in hematopoietic tissues normally used for clonogenic assays. The finding that EPA stimulates erythroleukemia cells suggests that EPA acts directly on neoplastic erythroid progenitors and that the effects are not mediated by products of interacting cell populations.

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Human leukemia cell line K562 responds to erythroid-potentiating activity

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