Purification and Characterization of Human T-lymphocyte-derived Erythroid-potentiating Activity*

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The human T-lymphoblast cell line, Mo, secretes a number of lymphokines, including erythroid-potentiating activity (EPA), an important early regulator of erythropoiesis. We report purification of EPA to homogeneity, from serum-free Mo-conditioned medium. Purification was accomplished by sequential concentration, ammonium sulfate precipitation, lentil lectin affinity chromatography, gel filtration, and reverse-phase high-performance liquid chromatography. EPA was assayed by its ability to stimulate the growth of large erythroid colonies (bursts) from normal human peripheral blood. The purified EPA has a molecular weight of 28,000 and appears as a single band when electrophoresed under reducing or nonreducing conditions. Purified EPA stimulates the growth of both early and late erythroid precursors from human bone marrow, as well as colony formation by the K562 human erythroleukemia cell line. Purified EPA has no colony-stimulating factor activity nor does it appear to be a structural protein of the human T-cell leukemia virus subtype II which infects the Mo cells.

EXPERIMENTAL PROCEDURES

Culture of the Mo Cells—To obtain large volumes of Mo-conditioned medium, cells were grown in T flasks containing about 200 ml of culture medium. Lots of 10 or 20 liters were prepared. Late passage Mo T-cells were used to prepare the conditioned medium because these cells are able to grow in the absence of bovine serum (13). Cells were grown for expansion at 37 °C in Iscove's modified Dulbecco's medium (Irvine Scientific) supplemented with 0.1% bovine serum albumin (Armour Pharmaceuticals, Reheis No. 2266-01, selected lots), 0.035% dextrose, 2.2 μM human transferrin (Sigma), antibiotics, and 1% glucose to a final density of about 2 × 10^6/ml. The cells were centrifuged and transferred to twice the original volume in a medium consisting only of Iscove's modified Dulbecco's medium containing antibiotics and glucose. They were incubated for an additional week and then removed by centrifugation. After collection, the medium was heated to 56 °C for 30 min to inactivate the HTLV-II.

Biologic Assay for EPA Activity—The method used to assay EPA is a modification of the previously described BFU-E assay (23–25). The peripheral blood leukocyte fraction (buffy coat) obtained from healthy donors was plated in a mixture consisting of 0.8% methyl cellulose (Dow Chemical Co.), 3.0% fetal bovine serum (Irvine Scientific and HyClone selected lots), 0.5 unit/ml of human urinary erythropoietin (supplied by the National Heart, Lung and Blood

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1 The abbreviations used are: EPA, erythroid-potentiating activity; HPLC, high-performance liquid chromatography; HTLV-II, human T-cell leukemia virus subtype II; BFU-E, burst-forming unit, erythroid; CFU-E, colony-forming unit, erythroid; PBS, phosphate-buffered saline.
Institute, about 1140 units/mg of protein), 1 μM FeCl₃, 4.4 μM human transferrin, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, 10⁻⁴ M α-thioglycollate, 10⁻⁷ M selenium, 0.05% dextrose, and Iscove’s medium. The assays were done in 96-well tissue culture plates (Costar); each well contained 0.1 ml of methylcellulose mixture with cells at a density of 4 to 5 × 10⁵/ml and 0.01 ml of test material. The material to be tested was dissolved in Dulbecco’s phosphate-buffered saline (Irvin Scientific) containing 0.01% bovine serum albumin and sterile filtered. 2-fold dilutions were made; each dilution was assayed in duplicate. Erythroid bursts were scored at 10 to 14 days using an inverted microscope. These bursts contain a minimum of 50 cells and are reddish brown in color indicating hemoglobinization of the cells. Typically, control wells contained approximately 30 erythroid bursts per well. The last dilution which gave stimulation greater than 30% above control was considered to have 1 unit/ml of EPA.

Other Bioassays for EPA—BFU-E were also assayed from normal human bone marrow by a previously described technique (23, 25); light density bone marrow cells were plated at 3 × 10⁵ cells/ml in Iscove’s medium containing 10% fetal bovine serum, 1 unit/ml of human erythropoietin, 0.8% methylcellulose, and antibiotics. Human bone marrow CFU-E were enumerated at 7 days in 1-ml gridded plates containing 2 × 10⁴ bone marrow cells/plate (23, 25). Colony forming units by K562 erythroleukemia cells was analyzed as described (17). The growth of human bone marrow granulocyte-macrophage colonies was assayed as described previously (27).

Purification of EPA—All steps except HPLC were carried out at 4°C. After lentil lectin chromatography, manipulations were performed in polypropylene tubes or siliconized glassware to minimize loss by adsorption.

Phenylmethylsulfonyl fluoride was added to 10 liters of conditioned medium at a final concentration of 1 mM to inhibit proteolysis. The media was then concentrated 15-fold with an Amicon hollow-fiber apparatus, using a Type H1P9-8 filter. The concentrate was brought to 80% saturation by the addition of solid ammonium sulfate. The precipitate was collected after stirring on ice for 4-12 h, dialyzed against PBS, and clarified by centrifugation. This material was applied to a column (2.6 × 6 cm) of lentil lectin-Sepharose 4B (Pharmacia) that had been equilibrated with PBS. The column was then washed with 2 column volumes of buffer, and the EPA was eluted with 15 ml of 0.5 M α-methyl-D-mannoside in PBS. The eluate was collected and concentrated in an Amicon stirred ultrafiltration cell, using a PM10 membrane, to a volume of 6 ml. The material was then applied to an Ultrogel AcA 44 column (1.6 × 80 cm; LKB) equilibrated with PBS as the running buffer. The flow rate was 6 ml/h, and fractions of 2.5 ml were collected. Fractions with the highest specific activity were combined and applied directly to the HPLC column without additional manipulation. Reverse-phase high-performance liquid chromatography was performed on a Beckman apparatus, using a Vydac C-18 column (10 × 250 mm) with a starting solvent of 10% acetonitrile and 0.1% trifluoroacetic acid in water. The column was eluted with increasing concentrations of acetonitrile in 0.1% trifluoroacetic acid using the following gradient: 10-30% acetonitrile over 10 min, then 30-60% over 60 min. The flow rate was 1.5 ml/min. Ultraviolet absorbance was monitored at 220 nm, and protein peaks were collected manually, evaporated to dryness, and reconstituted in a small volume of PBS.

Protein Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (28). Proteins were visualized by iodoion prior to electrophoresis. Iodinations were performed using a modification of the chloramine-T method (29); after reaction with [125I] and chloramine-T, the samples were not decontaminated by gel filtration but were applied directly to the gel. The tracking dye was electrophoresed to the end of the gel. This procedure obviated the need for the addition of carrier protein and allowed complete recovery of small amounts of protein. The gels were fixed in 50% methanol, 10% acetic acid, soaked in 2% glycerol, dried, and exposed to film for 1-12 h. In some experiments, proteins were visualized by staining with silver staining (30).

Protein Determination—Protein concentration was measured by the Bio-Rad method.

RESULTS

Bioassay of EPA—A typical dose-response curve for partially purified EPA is presented in Fig. 1. EPA was active over a narrow concentration range, generally less than two logs. A steep dose-response effect was seen, with activity rapidly reaching a plateau beyond which stimulation was not observed. At higher concentrations, activity was generally observed to decrease, sometimes falling to control levels. Even purified EPA demonstrates a decrease in activity at high concentrations. We have found that a decline in stimulation at high concentration is a property of many hormones which stimulate BFU-E growth, including growth hormone (26), insulin (24), and placental lactogen (23); it is not clear whether this behavior is an artifact of the bioassay system or is physiologically important. Additionally, EPA from earlier purification steps (ammonium sulfate precipitation and lentil lectin eluate) was frankly inhibitory at moderate concentrations. Because of these considerations, it was necessary to assay EPA over a wide concentration range for accurate quantitation. The addition of 0.01% bovine serum albumin to the dilutions was necessary to prevent loss by nonspecific adsorption, especially during sterile filtration. Bovine serum albumin has a slightly stimulatory effect in the assay; however, this effect was negligible at the concentration (0.01%) used.

Purification—A summary of a typical purification of EPA from 10 liters of conditioned medium is presented in Table I. It was difficult to accurately measure the amount of EPA in crude material and during the early steps of purification because of the presence of inhibitors of BFU-E growth. Although phenylmethylsulfonyl fluoride was routinely added to inhibit proteolysis, it may be unnecessary since the yields did

![Dose-response curve for partially purified EPA](image-url)
not appear lower when it was omitted. In general, about 1/5 - 1/4 of EPA flows through the lentil lectin Sepharose without binding. The flow-through activity may represent molecules which lack the carbohydrate moieties necessary for binding to the lectin, as has been described for other lymphokines such as colony-stimulating factor (3). We generally observed a loss of activity at this stage, and it appears to be due to the inhibitory nature of the column eluate. Gel filtration in Ultrogel was an important step in the purification of EPA which eluted from the column with an apparent molecular weight of 28,000 (Fig. 2). Last, reverse-phase HPLC was performed (Fig. 3). Manual collection of protein peaks allowed better resolution of the protein components and yielded a homogeneous preparation of EPA.

The overall purification resulted in an apparent yield of 25%; however, this figure is likely an overestimation due to inhibitors present in the crude material. Generally, approximately 25 μg is recovered from 10 liters of serum-free medium. The final product had a specific activity of 60 units/mg of protein, indicating that it is biologically active in the picomolar range. EPA retains activity over several months when stored at 4 °C.

Analysis of Purity and Molecular Weight Determination—Highly purified 131I-labeled EPA was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels (Fig. 4). The protein migrates as a single broad protein band (characteristic of many glycoproteins) at a molecular weight of 28,000 in the presence (lane A) or absence (lane B) of 10% β-mercaptoethanol, suggesting that it exists as a single polypeptide chain. A single band was observed with either silver staining or autoradiography of 131I-labeled material. Attempts to elute the activity from gel slices by the method of Burgess et al. (31) yielded ambiguous results. Although activity appeared to coincide with the main protein fraction the assays were difficult to interpret because of the extreme variability of background activity due to the presence of BFU-E inhibitors in the gel eluates. When highly purified biologically active EPA was analyzed again by reverse-phase HPLC, a single protein peak was observed (Fig. 5). Finally, approximately 10 μg of this purified material was analyzed in an automated microsequenator, and 11 cycles revealed single amino acids, confirming the presence of a homogeneously purified protein.

Characterization of Purified EPA—Serial dilutions of purified EPA were assayed for their ability to stimulate growth of peripheral blood BFU-E (Fig. 6). The shape of the dose-response curve is very similar to that seen using partially purified EPA (see Fig. 1). Maximal stimulation is seen at a dilution corresponding to a final concentration of EPA on the order of 10 pM. Similar results are seen using normal human bone marrow as the source of BFU-E (data not shown).

Partially purified EPA has previously been shown to stimulate human bone marrow CFU-E (6) and colony formation by K562 erythroleukemia cells in semisolid medium (17). Purified EPA is active in these assays (Fig. 7) in the same range of concentrations used to stimulate human peripheral blood and bone marrow BFU-E. These results demonstrate that a single purified molecule, EPA, is capable of stimulating the growth of both primitive human erythroid progenitors (BFU-E) and more mature erythroid precursors (CFU-E).

The Mo cell line elaborates a colony-stimulating factor that stimulates colony-forming cells of the granulocye-macro-

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3 R. Hewick, personal communication.
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FIG. 4. Molecular weight determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified 125I-labeled EPA (Fig. 3, fraction E) was boiled for 5 min in sodium dodecyl sulfate sample buffer with (lane A) and without (lane B) 10% β-mercaptoethanol and fractionated on a sodium dodecyl sulfate-polyacrylamide gel (10%). The dried gel was exposed to film. 14C-labeled molecular weight markers are myosin (200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa).

FIG. 5. Analysis of purity by HPLC. Fraction E (Fig. 3) was evaporated to dryness and resuspended in PBS. One-tenth of the sample as re-applied to the column and eluted with an acetonitrile gradient of 20-65% over 30 min. The upward slope of the baseline is due to the greatly increased sensitivity of the UV monitor as compared to Fig. 3.

Purification EPA was tested for colony-stimulating factor activity by its ability to support growth of human marrow granulocyte-macrophage colonies (27). When 1 × 10⁶ nonadherent human bone marrow cells were incubated with Mo-conditioned medium, approximately 50 granulocyte-macrophage colonies/plate were seen. When the conditioned medium was replaced by dilutions of purified EPA as shown in Figs. 6 and 7, no colonies were seen. Likewise, purified EPA was not active in bioassays that measure macrophage migration-inhibitory factor or neutrophil migration-inhibitory factor, lymphokines (also present in Mo-conditioned medium) which are thought to concentrate macrophages, and neutrophils at sites of inflammation (21, 22).

DISCUSSION

Under proper in vitro conditions, primitive erythroid precursors (BFU-E) give rise to colonies made up of thousands of hemoglobinized cells. By measuring the ability of various materials to enhance the formation of erythroid bursts, it has been possible to identify substances from murine and human sources that promote the growth of these early progenitors. We have purified one such burst-promoting activity, EPA, to homogeneity from conditioned medium of the Mo cell line.

Erythroid-potentiating activity has been demonstrated in the pellet obtained by high speed centrifugation of Mo-conditioned medium (the fraction which contains membrane vesicles) (32). This fraction also contains HTLV-II virions (12, 13). Furthermore, most cell lines infected with HTLV-II produce a burst-promoting activity (9, 10). Thus, it seemed possible that EPA might be a viral protein. In order to test this hypothesis, several immunoprecipitation experiments were performed. We found that serum from the patient Mo (12) immunoprecipitated iodinated viral p24 prepared from...
virions isolated from Mo-conditioned medium, but did not recognize purified EPA. Conversely, two rabbit antisera directed against purified EPA immunoprecipitated iodinated EPA, but no iodinated virus proteins were precipitated. These results suggest that EPA is not a viral structural protein, but do not exclude the possibility that EPA is coded for by one of the open reading frames identified at the 3' end of the human T-cell leukemia virus (ATLV) genome (33); however, this possibility is unlikely since EPA is produced by normal phytohemagglutinin-treated lymphocytes.

Previous results showed that EPA can be partially separated biochemically from granulocyte-macrophage colony-stimulating factor activity which is also elaborated by the Mo cell line (14, 15). Results presented here, using homogeneous EPA, confirm these earlier findings. No bone marrow-derived granulocyte or macrophage colonies were seen when purified EPA was assayed over a wide range of concentrations. Interestingly, small clusters of 15–30 tightly packed, rounded cells were observed in the presence of EPA. These clusters could consist of primitive myeloid precursors which divide but are unable to differentiate in the absence of specific hemopoietics such as colony-stimulating factor and erythropoietin.

Purified EPA retains all of the activities tested that were attributed to the partially purified material. The formation of erythrocyte bursts from human peripheral blood and bone marrow is maximally stimulated at concentrations of 10–20 pm; this concentration is within the range seen for biologic activities of peptide hormones. As was noted earlier with partially purified preparations, purified EPA also stimulates growth of the more mature human CFU-E (5, 6); neither human nor murine burst-promoting activity stimulates mouse CFU-E which has been isolated from the EL-4 mouse lymphoma cell line and WEHI-3 mouse myelomonocytic cells (34–36). Interleukin-3 is a glycoprotein of 28,000 molecular weight (37, 38). In addition, purified burst-promoting activity from leukemic cell line and WEHI-3-conditioned medium stimulated multipotent stem cells, as well as committed erythroid precursors (19). These findings suggest that human EPA may be analogous to murine interleukin-3 and burst-promoting activity.

Results presented here demonstrate that purified EPA stimulates the growth of both primitive and more mature erythroid precursors. The availability of purified material will make it possible to determine what (if any) role EPA plays in the proliferation and differentiation of the earliest hemopoietic precursors.

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