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Erythropoietic Enhancing Activity (EEA) Secreted by the Human Cell Line, GCT

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Medium conditioned by the monocyte-like cell line GCT contains colony-stimulating activity (CSA), a mediator of in vitro granulopoiesis. Also, the conditioned medium (CM) contains erythroid-enhancing activity (EEA), which can be demonstrated in a system utilizing either nonadherent marrow or blood mononuclear cells, erythropoietin (1–2 units/ml), and 20 ml/dl fetal calf serum. Under these conditions, GCT CM enhances the growth of CFU-E and BFU-E. Attempts were made to characterize the molecular features of EEA. Serum-free GCT cell CM was fractionated on Sephacryl S200 and Ultrogel AcA54. EEA and CSA cochromatographed with apparent molecular weights of ~ 40,000 daltons on Sephacryl and ~ 30,000 daltons on Ultrogel. Fractionation on DEAE Sephacel led to an apparent separation of CSA from EEA; however, when diluted, the fractions containing CSA had EEA. Undiluted fractions containing potent CSA inhibited erythropoiesis; however, dilution of these fractions resulted in marked EEA. Diluted crude GCT CM and DEAE Sephacel fractions enriched in EEA were also capable of sustaining BFU-E in liquid culture and mediating erythropoietin-independent colony growth. CSA could not be unequivocally separated from EEA on concanavalin A-Sepharose, since the diluted void volume containing CSA also had EEA. EEA was present in CM boiled for 60 minutes, whereas CSA was markedly reduced but not abolished. The inverse relationship between CSA concentration and EEA mandates dilution of fractions when bioassayed for these two activities. Although CSA and EEA are similar in molecular weight, they appear to be partially separable by ion-exchange chromatography and heat stability.

Key words: erythropoiesis, granulopoiesis, colony stimulating factors, hematopoiesis, erythroid cell-growth

Recently, a two-regulator model of erythropoiesis was described [1]. This model proposes that early stages of erythropoiesis are controlled by a factor other than erythropoietin, whereas the latter is required for the terminal maturation of erythroid precursors. In vitro studies of early erythropoiesis have shown that erythroid burst-forming units (BFU-E) require a mediator termed "erythroid enhancing activity" (EEA) [2], "burst-promoting activity" (BPA) [1], or "burst-feeder activity" [3]. This activity has been

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described for both mouse and human BFU-E, and it is present in serum [1, 4] and in medium conditioned by peripheral blood leukocytes [2], non-irradiated [5] or irradiated [3] bone marrow cells, mitogen-stimulated mononuclear cells [1, 6, 7], normal or transformed T lymphocytes [8, 9], and monocytes and macrophages [10]. All of these sources of EEA also contain colony-stimulating activity (CSA), which is necessary for the growth of granulocyte-monocyte progenitors in viscous culture [2, 11, 12].

The human monocyte-like cell line GCT elaborates potent CSA for man and other species [13, 14]. We have found that EEA is also present in medium conditioned by GCT cells, and in this report we describe our efforts at fractionation of this activity. The importance of diluting elution fractions in measuring the biological activity of EEA is also emphasized.

METHODS

Cell Preparation

Bone marrow cells were obtained by 0.5–1 ml aspirates from normal human volunteers. Red cells were sedimented in 4.5 g/dl dextran, and residual nucleated cells were exposed to plastic surfaces in a two-step manner as described by Messner [15]. The following modifications were made: cells were diluted to a 50 ml volume in McCoy's 5A with 10% (v/v) fetal calf serum and placed in 150 cm^2 Falcon plastic flasks for adherence. This adherence procedure resulted in no cluster or colony formation when 1×10^5 nonadherent cells were plated in the absence of CSA, even with 30% fetal calf serum.

Blood mononuclear cells were obtained from plateletpheresis residues and prepared as previously described [16]. Monocytes were depleted by iron filing adherence prior to Isopaque Ficoll density centrifugation and overnight adherence in 150 cm^2 Falcon plastic flasks. Blood cells were cultured at 2.5×10^5 ml.

Viscous Culture Technique

An 0.8 g/dl methylcellulose matrix was used for cell culture, as described previously [16], but with the following modifications: both granulocytic and erythroid progenitors were cultured with the same lot of fetal calf serum (20 ml/dl) and Iscove's modified Dulbecco's MEM without mercaptoethanol was used instead of Alpha MEM with mercaptoethanol. Bovine serum albumin (1.0 g/dl) was included in the cultures, and all incubations were at 5% CO_2, 37°C, 100% humidity. Bone marrow assays were done at 1×10^5 nonadherent cells per ml and blood cells at 2.5×10^5 per ml.

Erythropoietin, 2 units/ml (step III of Connaught), was used in all EEA assays unless otherwise specified. All CFU-C assays were done by adding 10% (v/v) of dialyzed, unconcentrated GCT cell-conditioned medium (CM) prepared as previously described [13]. Fractionation studies were made using serum-free GCT CM. The latter was prepared from GCT cells grown in McCoy's 5A medium containing 0.01 g/dl polyethylene glycol 6000 instead of calf serum as described previously [14].

CM fractions from DEAE and Ultrogel columns were usually diluted 1:3 in McCoy's 5A containing 5 ml/dl fetal calf serum prior to EEA and CSA assays.

CFU-E colonies were counted at day 7; BFU-E colonies were counted at day 14. Both were identified by the red color imparted by hemoglobinization. CFU-C colonies were scored at days 7 and 14, using 20 cells as minimum colony size.

Suspension Cultures

Nonadherent marrow cells were cultured in Falcon tissue culture tubes at 1×10^6/ml in McCoy's 5A containing 20 ml/dl fetal calf serum. Test fractions were added at 10 ml/dl to duplicate tubes, and incubation was carried out at 5% CO_2, 37°C for a period of 5–6 days. Prior to assay, cells were pelleted, supernates were discarded, and pellets were resuspended to the original volume of 1 ml. Aliquots of 0.1 ml were used for assay of EEA or CSA.

RESULTS

Effects of GCT Conditioned Medium on Erythroid Colonies

Medium conditioned by GCT cells consistently produced an enhancing activity for the human erythroid progenitors, CFU-E and BFU-E as well as potent CSA. The effect of GCT cell CM on blood BFU-E growth at different serum concentrations is shown in Figure 1. When blood mononuclear cells depleted on monocytes were plated at a cell density of 2.5×10^5ml, enhancement of BFU-E growth was observed at each concentration of fetal

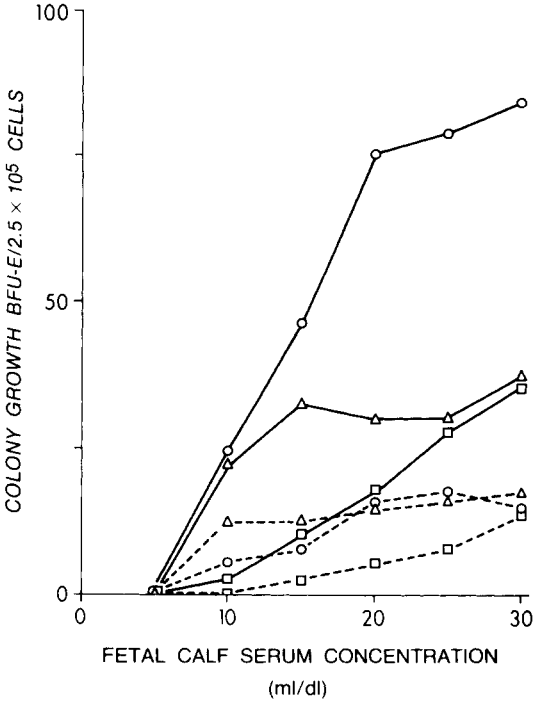

Fig. 1. Effect of dialyzed GCT conditioned medium on the fetal calf serum required for blood BFU-E growth. Solid lines represent cultures with 10% (v/v) CM; dashed lines, those without CM. The mean of duplicate observations for three experiments with and without CM are depicted by the paired symbols. Erythropoietin 2 units/ml was used in all cultures. Similar results were obtained with serum-free GCT conditioned medium.

calf serum tested. Bone marrow BFU-E growth also was enhanced by GCT CM. In all subsequent studies, the same fetal calf serum lot was used at a concentration of 20% (v/v) and erythropoietin at 2 units/ml in both CFU-E and BFU-E assays.

We examined the effect of GCT CM concentration on the growth of bone marrow CFU-E and BFU-E (Fig. 2a,b). The mean CFU-E in the absence of added GCT CM was 59 colonies. The optimum enhancement of CFU-E was at 2.5 ml/dl CM. Higher concentrations reduced, and eventually abolished, all enhancing activity. In some experiments growth was reduced below that of controls. A similar optimum was observed for marrow BFU-E. Thus, EEA was no longer seen when CM concentration was high.

GCT cells remain viable in serum-free medium fortified with polyethylene glycol [14]. Under these conditions they elaborate potent CSA. EEA was also present in serum-free GCT conditioned medium. Serum-free CM was used in the fractionation of EEA, which minimizes the interference of serum albumin in chromatographic analysis.

Chromatographic Fractionation of GCT Conditioned Medium

Ion-exchange chromatography. Initially, we placed concentrated serum-free CM through ion-exchange chromatography on DEAE Sephacel. The peak of the EEA eluted after the bulk (~90%) of CSA, when a linear gradient of sodium chloride was used (Fig. 3).

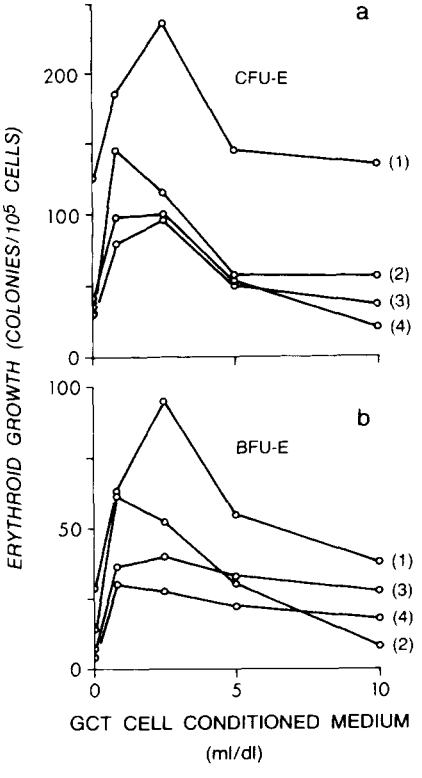

Fig. 2. Effect of dialyzed GCT conditioned medium on marrow CFU-E (a) and BFU-E (b). All cultures were made in 20% FCS with EPO 2 units/ml using 1×10^5 nonadherent cells. Numbers indicate paired experiments; mean of duplicate plates is shown.

CSA and EEA eluted between 0.1 and 0.2 M sodium chloride. We examined the peak CSA fraction (tube 155) and the peak EEA fraction (tube 170) for EEA at various dilutions (Table I). The fraction enriched for CSA contained EEA, which increased as the active fraction was diluted with control medium from 10% to 1%. The ratio of CSA/EEA was always higher on the ascending portion of the activity curves than on the descending limb, suggesting that the expression of EEA was more than simply a function of CSA concentration. The same elution profile was seen in four separate runs.

Gel filtration. In an effort to further discriminate between molecular species with weights in the 25,000–45,000 dalton range, we applied DEAE fractions containing CSA and EEA to Ultrogel AcA54 columns. DEAE fractions were pooled and concentrated by Amicon ultrafiltration with a PM 10 membrane. The elution profiles of CSA and EEA are shown in Figure 4. Both EEA and CSA appeared to co-chromatograph with a molecular weight of about 30,000 daltons. In studies not shown using Sephacryl S-200 chromatography, EEA and CSA eluted in similar fractions, with an apparent molecular weight of about 40,000 daltons.

In these experiments no EEA could be demonstrated in peak CSA fractions until dilution of samples was performed. EEA was tested, therefore, by adding 1.0% of the 1:3 dilutions of the Ultrogel fractions to cultures, whereas CSA was tested by adding 5%. When peak CSA fractions from the Ultrogel chromatography were tested at 10% no EEA could be demonstrated. Thus, EEA, which was present in the peak CSA fractions, could be detected only if dilutions were performed.

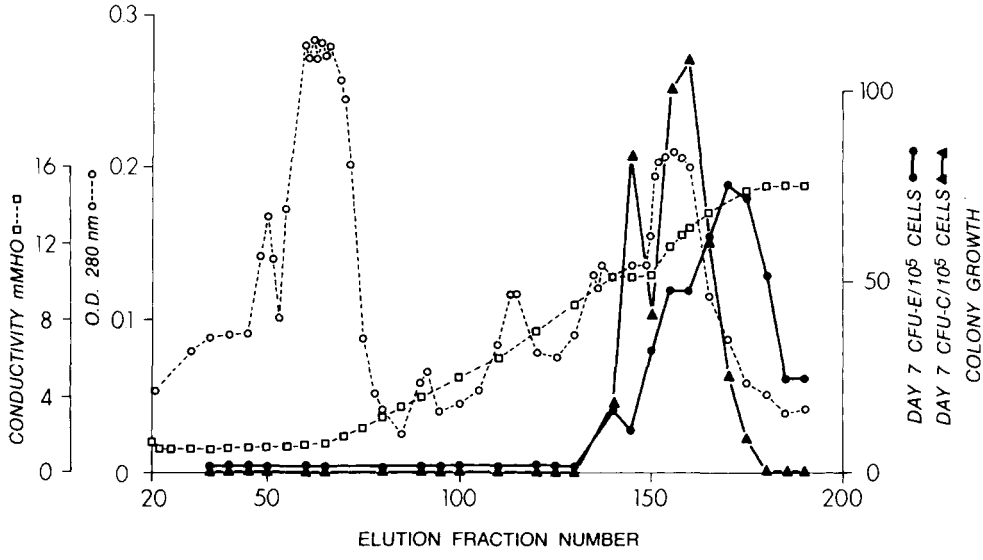

Fig. 3. DEAE Sephacel chromatography of a concentrated sample of serum-free GCT conditioned medium (3 liters) on a 5 × 80 cm column equilibrated in 0.03 M Tris-HCl buffer at pH 7.4 and eluted by a linear NaCl gradient from 0 to 0.25 M. All fractions were diluted 1:3 in McCoy's 5A with 5 ml/dl FCS and filtered prior to assay. CFU-C day 7 (closed triangles) and CFU-E day 7 (closed circles) were both cultured in 20% (v/v) FCS with a 10% (v/v) addition of each diluted fraction. Both CSA and EEA activities overlap, but the apparent peak of EEA elutes behind the peak of CSA. A similar elution profile was seen whether day 7 or day 14 colonies were assayed.

Affinity chromatography. In an effort further to separate EEA and CSA, we hoped to capitalize on a preferential binding to lectin by one or the other material. Therefore, we applied serum-free GCT CM to concanavalin A Sepharose 4B. As shown in Figure 5, CSA and EEA were partially bound to the lectin and were eluted by α-methyl mannoside; however, both CSA and EEA were present in the unbound fraction. When these fractions were pooled and rechromatographed on the same column, no further binding occurred, ruling out overloading as an explanation for the presence of both EEA and CSA in the unbound fractions. In other experiments we found that when the unbound fraction containing CSA was pooled prior to assay, EEA activity was present only when dilutions were carried out. Hence, in order to rule out the presence of EEA in unbound fractions, one had to test diluted fractions to ensure that inhibition did not occur from potent CSA.

Effect of Boiling on CSA and EEA in GCT Conditioned Medium

Golde et al [9] reported on the dissociation of EEA and CSA by boiling Mo cell line conditioned medium. We investigated the effect of varying times of boiling up to 60 min on EEA and CSA, both assayed in identical systems except for the EPO added to EEA assays (Fig. 6). EEA was present in GCT conditioned medium boiled for up to 60 min, and its apparent activity increased in tandem with a reciprocal decrease in CSA, both activities

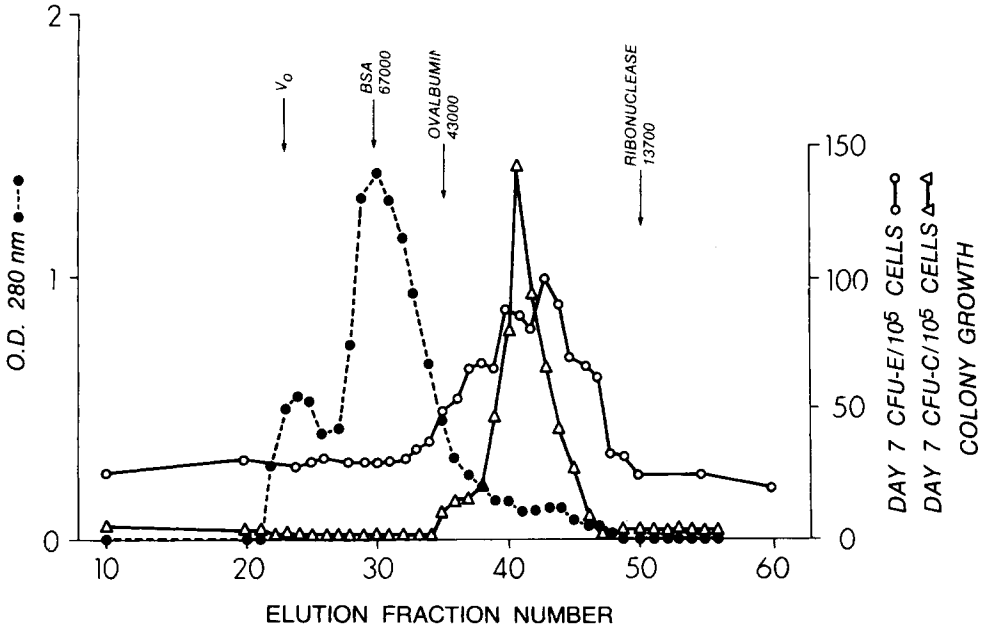

Fig. 4. Ultrogel AcA54 elution profile of serum-free GCT conditioned medium. DEAE Sephacel active CSA-EEA pools representing ~ 10 liters of medium were concentrated to 10 ml by ultrafiltration. A sample of 6 ml was applied to a 2.5 × 10 cm Ultrogel column in 0.05 M Tris HCl buffer, pH 7.4, 0.1 M NaCl, 0.01% PEG 6000, and eluted at 0.5 ml/min . Ten-ml fractions were diluted 1:3 prior to testing for EEA at 10 μl/ml EPO 2 units/ml, or CSA at 50 μl/ml in the same system without EPO. Both CSA and EEA peaks eluted with apparent weights of ~ 30,000 daltons as judged by assay of day 7 colonies. A similar profile was obtained when day 14 colonies were assayed.

reaching plateaus after 10 min. CSA was never completely abolished, since up to 25 small colonies/10^5 cells were obtained even after 60 min of boiling.

Effect of GCT CM on Erythropoietin-Independent Growth in Liquid Cultures

GCT conditioned medium was capable of sustaining BFU-E growth in viscous culture when erythropoietin addition was delayed for two days after cultures were initiated (Fig. 7). A similar sustaining action of GCT CM on marrow BFU-E was demonstrated when cells were preincubated in liquid suspension cultures for up to six days prior to colony assay (Table II). Enhancement of erythroid colonies occurred at the lowest dilutions of fractions containing CSA. This was associated with an increase in CFU-C as well. In control tubes BFU-E number dropped after the six days of incubation prior to assay.

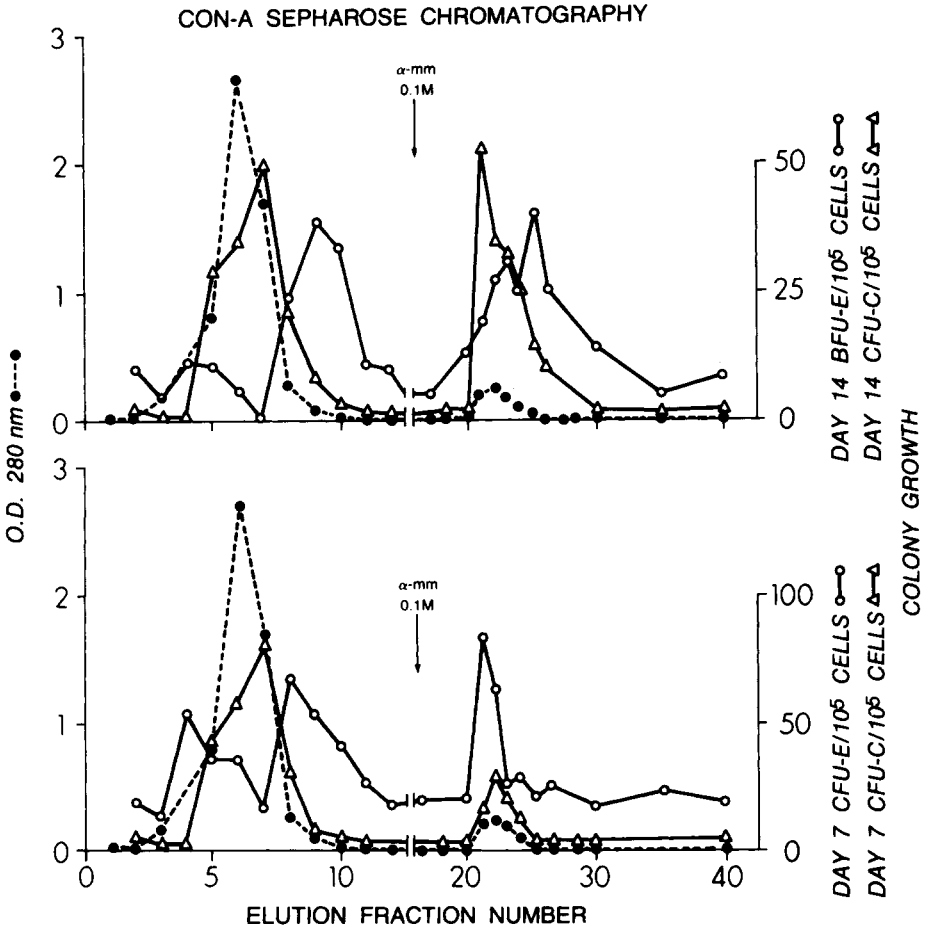

Fig. 5. Affinity chromatography of serum-free GCT conditioned medium. One liter was concentrated to 25 ml and applied to a 2.5 X 30 cm concanavalin A Sepharose column equilibrated in Tris-HCl, 0.05 M, pH 7.4; NaCl, 0.5M; polyethelene glycol, 0.05%; and $CaCl_2$, $MgCl_2$, $MnCl_2$ 0.1 mM at 4°C. α-methyl mannoside (0.1 M) in the same buffer was used for the elution. Fractions were dialyzed against 0.05 M Tris HCl pH 7.4, before assay, at 10% v/v. CSA and EEA were present in unbound fractions as was the fraction eluted with α-methyl mannoside.

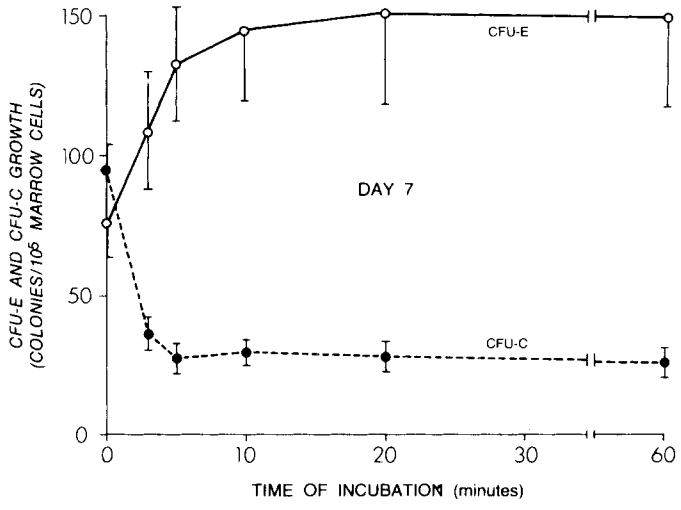

Fig. 6. Effect of boiling on CSA and EEA in dialyzed GCT conditioned medium. Test fractions were added at 10% (v/v) in cultures containing 20 ml/dl fetal calf serum, 2 units/ml erythropoietin, and 1×10^5 nonadherent marrow cells. A rapid decrease in CSA occurred after three minutes of boiling coincident with an increase in the number of erythroid colonies at 7 days. Similar results were seen with day 14 colonies. Mean + SE are shown, erythroid colonies in triplicate, granulocytic in duplicate, for two different experiments.

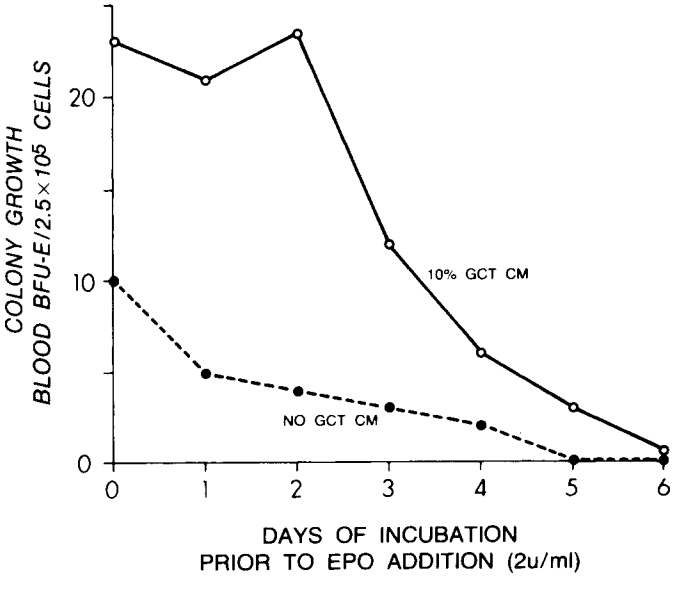

Fig. 7. Effect of GCT CM on BFU-E growth when erythropoietin addition is delayed. EPO 2 units/ml in 100 μl volume was pipetted gently over dish without disturbing the cultures. A similar effect was seen when marrow BFU-E were analyzed.

TABLE I. Effect of Dilution on EEA Activity in DEAE Fractions

DEAE Sephacel fractions	DEAE fractions added to culture (vol %)	Colonies/10^5 cells[a]	
		CFU-E	BFU-E
CSA peak (tube 155)	10	68	18
	5	82	26
	2.5	100	32
	1	120	30
EEA peak (tube 170)	10	96	27
	5	91	18
	2.5	50	6
Control medium	10	40	4

[a]All cultures stimulated by 2 units/ml EPO, FCS 20% (v/v). Data represent means of duplicate plates. DEAE fractions were all added at volumes of 10% by addition of control medium.

TABLE II. Effect of GCT Conditioned Medium on BFU-E and CFU-C Growth in Liquid Suspension After 6 Days of Incubation*

Test fraction	Day 14 BFU-E	% Control	Day 14 CFU-C	% Control
Control medium	22	100	49	100
GCT CM 20%	20	91	72	147
10%	27	123	78	159
1%	43	195	83	169
DEAE CSA peak (fraction 155) 10%	38	175	77	157
DEAE EEA peak (fraction 170) 10%	42	193	68	139
DEAE CSA pool (52–59) 1%	57	259	72	147

*Mean values of duplicate plates. Cells were centrifuged and reconstituted to 1 ml final volume with 0.1 ml added per ml culture. EPO 2 units/ml and FCS 20% (v/v) were included in assays. EPO was omitted in CSA assays. The day zero control contained 28 BFU-E and 50 CFU-C per 10^5 cells (measured at day 14 of viscous culture).

DISCUSSION

The description of an erythroid-enhancing activity in leukocyte conditioned medium was made initially by Aye [2], who used human marrow and blood cells thoroughly depleted of adherent cells. In parallel, evidence was accumulating that erythropoietin was not the sole regulator of the early BFU-E compartment in vivo from studies of mice subjected to hypertransfusion [1] and phenylhydrazine-induced hemolysis [3]. Subsequently EEA has been described in serum and conditioned media from various sources using normal or transformed cells [1–10].

Our studies describe the properties of EEA secreted by the GCT cells, a permanent culture derived from a malignant fibrous histiocytoma [13]. These cells elaborate, in addi-

tion to CSA, a low molecular weight inhibitor (CIA), which inhibits erythroid growth at low cell densities [14]; plasminogen activator (unpublished); lymphocyte activating factor (unpublished); and a factor responsible for the growth of primitive macrophage progenitors [19]. Both classes of erythroid progenitors, BFU-E and CFU-E, appear responsive to this mediator, indicating effects on very early, as well as later, stages of erythroid development. This finding is in agreement with the results obtained by Golde et al with EEA elaborated by the Mo cell line [9]. The active component in GCT conditioned medium containing EEA and CSA has an apparent molecular weight of about 30,000 daltons by gel filtration on Ultrogel AcA54. This value is somewhat lower than other estimates. Human-active EEA derived from the Mo cell line [9] and eluted from Ultrogel AcA44, or from leukocyte and placenta conditioned medium [17] with Sephadex G-100, had an apparent molecular weight of about 45,000 daltons. The active human CSA peaks from these other sources were around 30,000 daltons [9, 17]. These differences in molecular weight estimates of EEA and CSA by gel filtration can be reconciled if they represent differences in biochemical characteristics of EEA from various sources. Another explanation may reside in the fact that these authors [9, 17] used different bioassay conditions for CSA and EEA, with different matrices and fetal calf serum concentrations. The apparent separation of EEA from CSA by gel filtration may also be due to the inverse relationship of EEA expression and CSA titer. Hence, the CSA peak region around 30,000 daltons may still have EEA when dilutions of these fractions are made. This is similar to the observations of Van Zant et al [18] that describe reduced erythroid colony growth by high titers of purified mouse CSF and enhancement at low CSF concentrations.

Complete dissociation of CSA from EEA in GCT conditioned medium was not achieved by ion-exchange chromatography or boiling. Furthermore, we have shown partial binding of CSA and EEA to concanavalin A Sepharose. Aye [20] has reported that EEA from leukocyte-conditioned medium, unlike CSA, bound to con A Sepharose, but dilutions of the CSA peak were not reported to ensure the absence of EEA. Nevertheless, the finding that EEA elutes always after the CSA by ion-exchange chromatography suggests that these two entities may be separable.

The role of EEA in early erythropoiesis in vitro is underscored by the enhanced survival of BFU-E in delayed EPO addition experiments and in liquid suspension cultures. The relationship of EEA to CSA is at present unclear. Iscove and co-workers [17] have reported the presence of delta colony potentiating activity (ΔCPA), an activity sustaining CFU-C growth in suspension cultures, in their EEA elution fractions. Our results in suspension culture show a sustained proliferation of both CFU-C and BFU-E in diluted CSA fractions. This may indicate that EEA may affect the proliferation of primitive stem cells, regardless of their terminal pathway of differentiation. Alternatively, this could indicate that a granulocyte-enhancing activity is present in EEA fractions. Although it may appear physiologically unlikely that a single mediator would influence distinct hemopoietic lineages, proof of this contention awaits further purification. In this regard, studies by Metcalf et al [21] have suggested that highly purified mouse lung CSF can directly stimulate multipotential and early erythroid precursor cells in fetal liver cell cultures. These observations, as well as those of Van Zant et al [18], suggest enhanced erythroid growth by purified CSF. To answer this question beyond doubt, it will be necessary to quantitate CSA and EEA in the same preparations, preferably using identical assays in vitro.

The availability of human cell lines such as Mo and GCT should facilitate the characterization and purification of EEA and CSA from cell sources that elaborate factors similar to monocytes and T lymphocytes.

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