

# Regulation of Human Megakaryocytopoiesis

## AN IN VITRO ANALYSIS

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**ABSTRACT** We have recently described an assay system for human peripheral blood megakaryocyte colony-forming unit cells (CFU-M) using an anti-platelet glycoprotein antiserum probe to define megakaryocyte colonies grown in vitro. This system was applied to study the nature and regulation of human bone marrow CFU-M. In the absence of a specific megakaryocyte growth-promoting factor,  $12.4 \pm 3.0$  (means  $\pm$  SEM) megakaryocyte colonies were cloned per  $5 \times 10^5$  cells cultured. Colonies were present after 6 d of incubation reaching peak numbers between days 10 and 14 and slowly decreasing thereafter. Erythropoietin in concentrations of up to 4 U/ml failed to augment colony numbers. Also failing to enhance megakaryocyte colony plating efficiency were media containing burst-promoting activity and colony-stimulating activity. A medium conditioned by human embryonic kidney cells, which has been previously demonstrated to contain thrombopoietin, also had no effect on megakaryocyte colony numbers. In contrast, sera from three patients with severe aplastic anemia produced significant enhancement of CFU-M-derived colony formation in vitro. Both the number of megakaryocyte colonies present and the number of megakaryocytes per colony were increased in proportion to the final concentration of aplastic anemia serum. In the presence of 10% aplastic anemia serum, cultured megakaryocyte colony numbers were linear with respect to the number of bone marrow mononuclear cells plated suggesting a clonal origin of each of the colonies. This in vitro assay for bone marrow CFU-M is a reliable means by which to study the regulation of human megakaryocytopoiesis. Initial data suggest that mega-

karyocyte production is stimulated by a factor detectable in aplastic anemia serum that may be distinct from other known hematopoietic stem cell regulators.

## INTRODUCTION

The study of human megakaryocytic progenitor cells proved to be difficult because of the lack of a well-defined histochemical phenotypic marker to readily identify colonies composed of megakaryocytic elements. Previous studies of human megakaryocyte colony-forming cells (CFU-M, or colony-forming unit-megakaryocyte)<sup>1</sup> have depended upon morphologic criteria to identify the clusters of differentiated megakaryocytes cloned in vitro from peripheral blood and bone marrow CFU-M (1-3). We have recently established an assay system for human peripheral blood CFU-M using a rabbit antiserum to purified human platelet glycoproteins (PGP) as a phenotypic probe to define human megakaryocytic colonies grown in vitro (4, 5). In these studies, megakaryocyte colonies were successfully cloned from the peripheral blood of all normal volunteers studied. Plating efficiency was not enhanced by the addition of known in vitro hematopoietic stem cell regulators, i.e., erythropoietin, burst-promoting activity, and colony-stimulating activity (4, 5). In addition, a source of thrombopoietic stimulating factor or thrombopoietin did not alter CFU-M-derived colony formation in our experiments (4, 5).

We report here studies concerning the nature and regulation of human CFU-M derived from bone marrow mononuclear cells. Marrow CFU-M did not re-

<sup>1</sup> *Abbreviations used in this paper:* BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; CFU-GM, colony-forming unit-granulocyte, macrophage; CFU-M, colony-forming unit-megakaryocyte; E-CM, human endothelial cell-conditioned medium; HEKM, human embryonic kidney cell-conditioned medium; Meg-CSA, megakaryocyte colony-stimulating activity; PBS, 0.01 M phosphate-buffered saline, pH 7.2; PGP, human platelet glycoproteins; TC-CM, human lymphoblastoid cell line-conditioned medium.

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spond to the various hematopoietic regulators which had been previously shown to be ineffective in stimulation peripheral blood CFU-M. However, megakaryocyte colony-stimulating activity (Meg-CSA) was present in sera obtained from three patients with severe aplastic anemia. These sera, when added to the culture media, significantly augmented CFU-M plating efficiency and increased the mean number of component cells per megakaryocyte colony. Such activity was not present in any normal human serum specimen. These data suggest that the human CFU-M may be controlled, in part, by a previously undefined humoral regulator that appears to be distinct from those substances known to stimulate erythroid or granulocytic-macrophage colony formation in vitro. We were also able to demonstrate that after the addition of the stimulating serum to the cultures, megakaryocyte colony numbers were linear with respect to the number of bone marrow cells cultured supporting the hypothesis of a clonal origin for each of the megakaryocyte colonies.

## METHODS

**Human subjects.** Bone marrow aspirations were obtained, after informed consent, from the posterior iliac crest under local anesthesia from hematologically normal individuals. These individuals were either normal volunteers or patients with normal hematologic parameters undergoing bone marrow aspirations in the course of their clinical evaluations.

**Plasma clot megakaryocyte cultures.** Bone marrow mononuclear cells were obtained by Ficoll-Paque (specific gravity = 1.077 g/cm<sup>3</sup>, Pharmacia Fine Chemicals, Piscataway, Div. of Pharmacia Inc., N. J.) density centrifugation. The bone marrow aspirate was diluted 1:1 with  $\alpha$ -medium minus nucleosides (Gibco Laboratories, Grand Island, N. Y.), containing preservative-free sodium heparin at 20 U/ml and layered over an equal volume of Ficoll-Paque. Centrifugation was performed at 500 g for 25 min at 4°C in a Beckman model J-6B centrifuge (Beckman Instruments, Inc., Fullerton Calif.). The interface mononuclear cell layer was collected and washed with  $\alpha$ -medium minus nucleosides containing 2% fetal calf serum. Mononuclear cells at a concentration of  $5 \times 10^5$  cells/ml (unless otherwise stated) were cultured in 1-ml vol in 35-mm petri dishes. The plasma clot technique of McLeod et al. (6) was modified by the substitution of heat-inactivated human AB serum for fetal calf serum, and  $\alpha$ -medium minus nucleosides for NCTC-109 medium and Eagle's minimal essential medium with Hanks' balanced salt solution. All batches of normal AB serum were prescreened for comparable capacity to support megakaryocyte colony growth. In the final 1-cm<sup>3</sup> aliquot of each culture were the following supplements: minimal essential medium, nonessential amino acids (0.02 mmol/ml), L-glutamine (0.4 mmol/ml), and sodium pyruvate (0.2 mmol/ml). Culture dishes were incubated for 10–12 d (unless otherwise stated) at 37°C in a 100% humidified atmosphere of 5% CO<sub>2</sub> in air. Harvesting was performed by fixation in situ with methanol/acetone (1:3) for 20 min, washing with 0.01 M phosphate-buffered saline (PBS), pH 7.2, distilled water, and then air drying. Plasma clots were stored frozen at -20°C until immunofluorescent staining was performed.

**Culture additives.** Various substances were added to the culture growth media to assess their capacity to enhance in

vitro megakaryocyte colony formation. These consisted of sheep urinary erythropoietin (Epo, Connaught Laboratories Limited, Willowdale, Ontario, Canada, step III, lot 3032-1), human embryonic kidney cell conditioned medium (7) (HEKM, donated by Dr. T. P. McDonald), human endothelial cell conditioned media (8) (E-CM, donated by Dr. John C. Hoak), human T lymphoblastoid cell line conditioned medium (9–11) (TC-CM, donated by Dr. David Golde), and serum specimens from three patients with severe aplastic anemia.

The batch of erythropoietin used was active in stimulating erythroid colony formation in conventional plasma clot cultures as performed in our laboratory (12).

HEKM was received lyophilized, reconstituted with  $\alpha$ -medium minus nucleosides, and stored in small aliquots at -80°C until used. HEKM has been shown to have significant thrombopoietic activity as defined by increased Na<sub>2</sub><sup>35</sup> SO<sub>4</sub> incorporation into the circulating platelets of thrombocytotic animals (7).

E-CM is the supernatant medium derived from cultures of human umbilical vein and adult arterial endothelial cells obtained originally from intact vessels and cultured for 72 h in modified Medium 199 with 20% fetal calf serum as previously described (8). E-CM has been shown to contain products produced in vitro by human endothelial cells including prostacyclin (13), Factor VIII antigen (14, 15), and granulocyte-macrophage colony-stimulating activity (16, 17). When added to the modified plasma clot cultures in final concentrations of 10%, both adult artery and umbilical vein E/CM increased granulocyte-macrophage colony (CFU-GM) numbers by 50–100%.

TC-CM is medium conditioned by the cell line Mo that was established from the spleen cells of a patient with a T cell variant of hairy cell leukemia. This medium has been shown to augment erythroid burst colonies in methylcellulose cultures two- to threefold (9) and to have significant colony-stimulating activity (10, 11). When added to our modified plasma clot cultures in final concentrations of 10%, we observed augmentation of erythroid burst (in erythropoietin containing cultures) and CFU-GM colony formation similar in magnitude to that previously described.

Sera from three patients with severe aplastic anemia as defined by the International Aplastic Anemia Study Group (18) were obtained by routine venipuncture. The specimens were sterilized by ultrafiltration and heat inactivated at 56°C for 30 min, and added to the culture media by replacing an equal volume of normal human AB serum.

**Anti-PGP antiserum preparation.** Purified human PGP was prepared by lithium diiodosalicylate-phenol extraction of pooled human platelet concentrates as described by Marchesi and Chasis (19). New Zealand White rabbits were immunized by subcutaneous injections of 1 mg PGP in Freund's complete adjuvant initially and intramuscular injections (1 mg PGP in Freund's incomplete adjuvant) at 2 and 4 wk. Serum was harvested at 6 wk by cardiac puncture and stored in aliquots at -80°C.

**Immunofluorescent staining and scoring.** Whole rabbit anti-PGP antiserum was diluted in PBS (1:200) and layered over the fixed plasma clot cultures. These were incubated for 60 min at room temperature in 100% humidified air. After washing three times with PBS, the specimens were reincubated with fluorescein-conjugated goat anti-rabbit IgG (Meloy, Springfield, Laboratories, Inc. Va) diluted in PBS, final concentration 0.36 mg protein/ml for an additional 60 min. After washing with PBS, the specimens were counterstained with 0.125% Evan's Blue for 7 min, washed with distilled water and mounted in isotonic barbital buffer, pH 8.6, in glycerol (1:3).

In vitro plasma clot cultures were scored in situ. The 35-mm petri dishes were inverted, the base area completely scanned, and fluorescein-positive colonies enumerated with a fluorescence microscope at  $\times 100$  (Zeiss standard microscope 18 with IV FL vertical fluorescent illuminator, Carl Zeiss, Inc., New York). A megakaryocyte colony was defined as a cluster of three or more intensely fluorescent cells. Each study was performed in duplicate to quadruplicate.

**Other hematopoietic colonies.** Plasma clots were fixed in situ with 5% glutaraldehyde in 0.01 M phosphate buffer (pH 7.0–7.2). CFU-GM were assayed morphologically after Wright-Giemsa staining of the fixed cultures (20). Clusters of  $\geq 40$  cells, many of which had typical band or segmented polymorphonuclear leukocyte morphology, were defined as CFU-GM.

Colonies derived from the erythroid progenitor cells CFU-E (colony-forming unit-erythroid) and BFU-E (burst-forming unit-erythroid) were harvested after 7 and 12–14 d of incubation, respectively. Cultures were stained with 1% benzidine and hematoxylin as previously described (20). Erythroid colonies were scored using established criteria (12). Maximum growth of CFU-E and BFU-E-derived colonies in this modified plasma clot assay system was seen at erythropoietin concentrations of 2 and 4 U/ml, respectively. At these optimal concentrations of erythropoietin, means of 420 CFU-E- and 80 BFU-E-derived colonies were cloned from  $5 \times 10^5$  normal human bone mononuclear cells.

**Statistical analysis.** Results are expressed as the mean and SEM of the number of megakaryocytic colonies enumerated. Comparisons were made by paired one-tailed Student's *t* tests and linear regression analyses.

## RESULTS

In the absence of a specific growth-promoting culture additive, megakaryocytic colonies were successfully grown from the bone marrow mononuclear cells of 10 hematologically normal individuals.  $12.4 \pm 3.0$  (means  $\pm$  SEM) megakaryocytic colonies were cloned per  $5 \times 10^5$  cells cultured with plating efficiencies ranging from 2–32 colonies per  $5 \times 10^5$  cells cultured.

Individual megakaryocytes within a single colony generally were dispersed over an area approximately the size of an erythroid burst. Nuclear multilobulation was often evident in the component cells (Fig. 1). Some colonies consisted exclusively of or contained small fluorescein-labeled cells approximately the size of lymphocytes that were not otherwise morphologically recognizable as megakaryocytes. In the absence

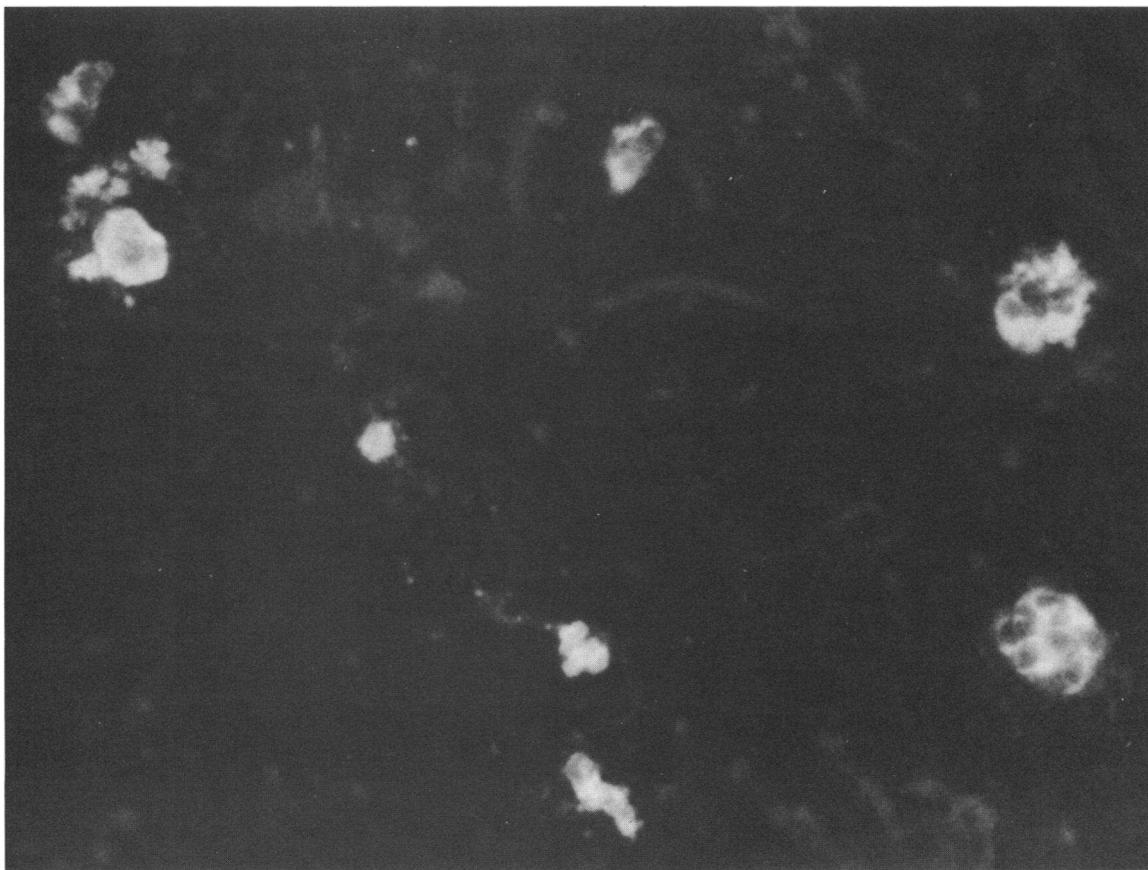


FIGURE 1 Single megakaryocyte colony in plasma clot culture labeled with antiplatelet glycoprotein antiserum and fluorescein-conjugated goat and anti-rabbit IgG cloned from human bone marrow mononuclear cells ( $\times 100$ ).

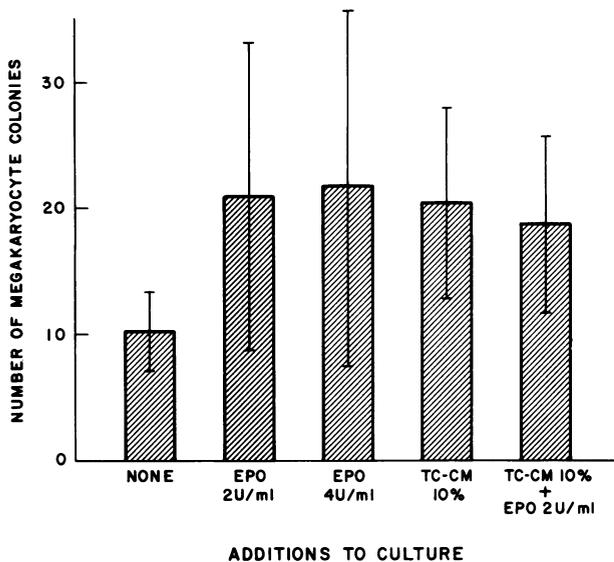


FIGURE 2 Effects of erythropoietin and TC-CM (with and without erythropoietin) on CFU-M-derived colonies cultured from human bone marrow mononuclear cells. Results expressed as mean  $\pm$  SEM for six experiments. The megakaryocyte colony numbers for erythropoietin and for TC-CM containing cultures (with and without erythropoietin) are not significantly increased above control cultures,  $P > 0.05$ . Epo, erythropoietin.

of culture additives, 35–75% of all the megakaryocyte colonies present consisted exclusively of these small fluorescein-labeled cells.

A variety of substances were tested for the capacity to enhance in vitro megakaryocytic colony formation. Sheep urinary erythropoietin in final concentrations of up to 4 U/ml failed to augment megakaryocyte colony numbers (Fig. 2).

HEKM, a source of thrombopoietic-stimulating factor or thrombopoietin (7), was similarly ineffective in enhancing megakaryocytic colony growth when added to the cultures in final concentrations of 75–600  $\mu$ g/ml of protein (Table I).

TABLE I  
Effect of HEKM on Number of Megakaryocyte Colonies Cloned\*

Experiment	HEKM concentration, mg protein/ml				
	0.0	0.075	0.15	0.30	0.60
1	11.5	11.5	11.5	7	6.5
2	5	ND	ND	6.5	ND
3	5.5	ND	ND	5.5	ND

\* Values are the mean colony numbers of experiments performed in duplicate with plated mononuclear cell concentrations of  $5 \times 10^5$  cells/ml. ND, not done.

TABLE II  
Effect of E-CM on Number of Megakaryocyte Colonies Cloned

Experiment	Culture additives				
	None	Adult artery-CM		Umbilical vein-CM	
		5%*	10%	5%	10%
1	3	1.5	2.5	3	3
2	7	3.5	4	3.5	3.5

\* Final concentration of media added. Values are the mean colony numbers of experiments performed in duplicate with plated mononuclear cell concentrations of  $5 \times 10^5$  cells/ml. CM, conditioned medium.

TC-CM, which has been demonstrated to contain burst-promoting activity (9) and colony-stimulating activity (10, 11), also failed to significantly increase the number of megakaryocytic colonies (Fig. 2) as did E-CM (Table II).

In contrast to the above results, serum specimens from three patients with severe aplastic anemia were found to reproducibly enhance CFU-M-derived colony formation when substituted for normal human AB serum in concentrations of 5–10% of the final culture volume (Table III). When higher concentrations of aplastic anemia sera were tested, the dose-response relationship was linear within final aplastic anemia serum concentrations of 5–30% (Fig. 3). Similar stimulatory activity was not present in any of eight normal serum specimens tested.

In addition to augmenting the total number of megakaryocyte colonies, all three aplastic sera increased the mean number of component cells per colony with a similar linear dose-response relationship (Fig. 4). For any given experiment, the relative intensity of augmentation of megakaryocyte colony numbers was paralleled by a comparable relative increase in the number of component megakaryocytes per colony (Figs. 3 and 4). The addition of aplastic anemia sera (10–30% final concentration) also resulted in a relative decrease in the colonies composed exclusively of small fluorescein-positive cells. Throughout this concentration range of sera, these small cell colonies comprised ~20% of the total number of colonies.

Aplastic anemia sera, constituting 10% of the final culture volume, did support some growth of erythroid colonies. In three experiments, a mean of 114 CFU-E- and 6-BFU-E-derived colonies were cultured per  $5 \times 10^5$  normal bone marrow mononuclear cells. These data are consistent with the increased levels of erythropoietin known to be present in aplastic anemia sera (21, 22). No augmentation of granulocyte-macrophage colony growth was seen with aplastic anemia serum concentrations of 5–30%.

TABLE III

Effect of Serum Specimens from Three Patients with Aplastic Anemia on the Number of Megakaryocyte Colonies Cloned\*

Experiment	Control	Final aplastic anemia serum concentration	
	0%	5%	10%
<b>Patient 1</b>			
1	10.5	48.5	70
2	2	26	38†
3	16	33.5	136.5
4	7	ND	129
5	3.5	15.5	69.5
6	11.5	ND	37
<b>Patient 2</b>			
1	16	36	64.5†
2	11	41	48
3	33	ND	75.5
4	11.5	ND	34.5
<b>Patient 3</b>			
1	28	63	60.5
2	16	27.5	65
3	12.5	ND	45
4	11.5	ND	80

\* Values are the mean colony numbers of experiments performed in duplicate with plated mononuclear cell concentrations of  $5 \times 10^5$ /ml. When compared with the control cultures each culture containing aplastic anemia serum demonstrates significantly increased colony numbers with  $P < 0.05$  or less (unless otherwise stated. ND, not done.

†  $0.10 > P > 0.05$ .

Because of the enhanced plating efficiency which could be achieved in the presence of aplastic anemia sera, additional analyses of the CFU-M assay system could be performed. These studies required larger numbers of megakaryocyte colonies per culture plate than could be otherwise obtained using normal AB serum alone.

Bone marrow mononuclear cells were plated in concentrations of  $0.5 \times 10^5$ – $5 \times 10^5$  cells/ml in the presence of 10% aplastic anemia serum. There was a linear relationship between the number of cells plated and the number of CFU-M-derived megakaryocytic colonies cultured. The Y intercept was approximately equal to zero in all experiments. Three representative experiments are displayed in Fig. 5. These data support the hypothesis of a clonal origin of each of the megakaryocytic colonies.

The chronology of the development of megakaryocytic colonies in vitro was evaluated in the presence of 10% and 20% final concentrations of the aplastic anemia serum. The two concentrations of serum were chosen to detect temporal alterations which might have been attributable to the presence of the aplastic

anemia serum in the culture system. Megakaryocyte colonies were detectable after 6–7 d of culture reaching a maximum between 10 and 14 d and slowly decreasing thereafter (Fig. 6). Though megakaryocytic colony numbers were greater in the presence of 20% aplastic serum as compared with 10%, there was no significant change in the time of colony appearance (data not shown).

## DISCUSSION

Our present understanding of megakaryocytopoiesis has been derived in a large part from detailed and painstaking analyses of morphologically recognizable megakaryocytes present in bone marrow specimens from experimental animals and patients with normal and perturbed thrombopoiesis (23). We now know, however, that this polyploid, morphologically identifiable megakaryocyte compartment includes only cells

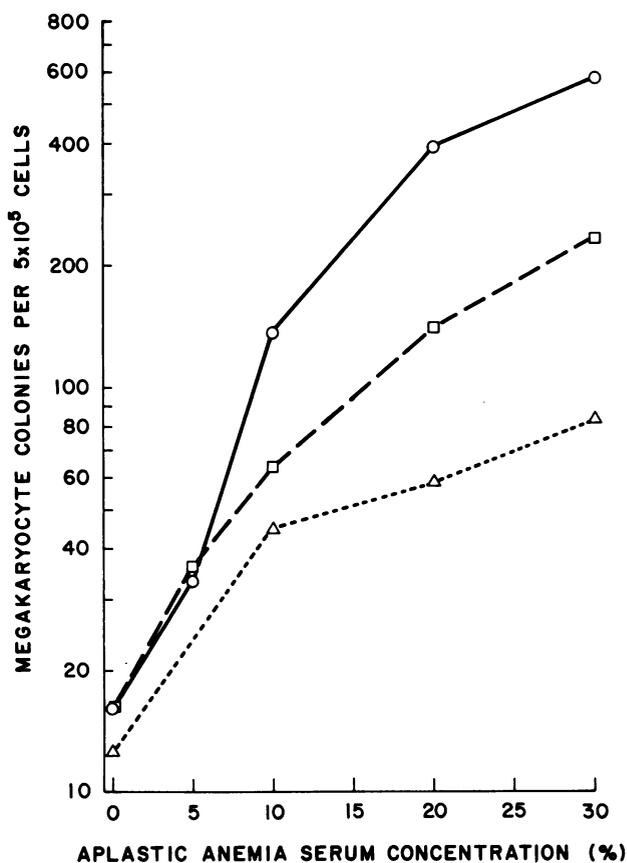


FIGURE 3 Effects of the concentration of sera from three patients with aplastic anemia on the number of CFU-M-derived colonies cultured from human bone marrow mononuclear cells. Each line is derived from a single representative experiments performed in duplicate. A proportional dose-response effect is demonstrated in each experiment. Patient 1,  $\circ$ ,  $r = 0.817$ ,  $P < 0.01$ ; patient 2,  $\square$ ,  $r = 0.980$ ,  $P < 0.01$ ; patient 3,  $\triangle$ ,  $r = 0.978$ ,  $P < 0.01$ .

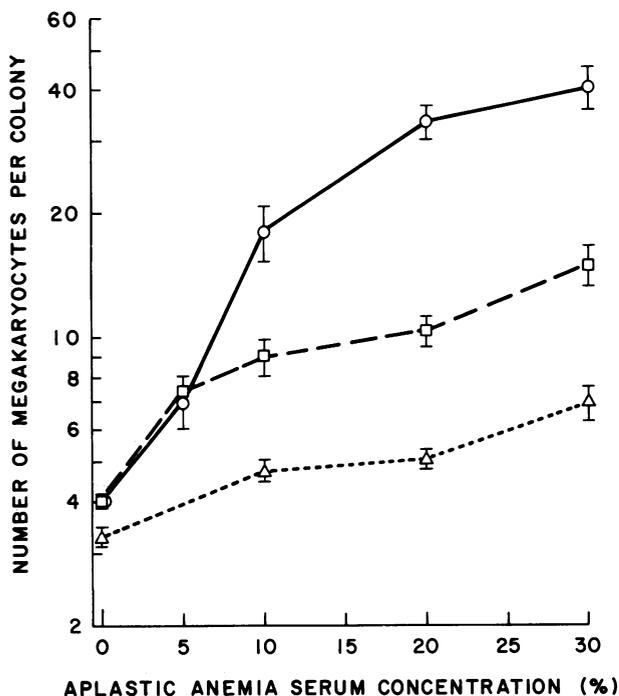


FIGURE 4 Effects of the concentration of sera from three patients with aplastic anemia on the number of megakaryocytes present in individual megakaryocyte colonies. Each point represents the mean  $\pm$  SEM number of megakaryocytes per colony derived from examining 50 colonies cultured during a single experiment at different aplastic anemia serum concentrations. At 0% aplastic serum concentration, the number of colonies examined varied from 38–50 due to the limited colony numbers present. Data for these three experiments are derived from the same experiments presented in Fig. 3. In each experiment, a proportional dose-response relationship is present between the mean number of megakaryocytes per colony and the concentration of aplastic anemia serum. Patient 1,  $\circ$ ,  $r = 0.985$ ,  $P < 0.01$ ; patient 2,  $\square$ ,  $r = 0.975$ ,  $P < 0.01$ ; patient 3,  $\triangle$ ,  $r = 0.967$ ,  $P < 0.05$ .

undergoing the terminal stages of megakaryocyte maturation and platelet release. Regulatory schema of megakaryocytopoiesis have been predominantly limited to explaining observations that occur in this nonproliferating and relatively differentiated cellular compartment whereas the proliferative events, occurring at an earlier stage of differentiation, can only be indirectly inferred.

It is now generally agreed that megakaryocytes originate from a pluripotent hematopoietic stem cell that in turn gives rise to proliferative, nonrecognizable megakaryocyte-committed progenitors capable of supplying cells to the recognizable megakaryocyte compartment (24).

Rodent *in vitro* clonal assay systems have been recently established in which colonies of megakaryocytes can be cloned from these megakaryocyte-committed stem cells or CFU-M (6, 25–29). The mega-

karyocytic identity of the colonies is often confirmed or established using a histochemical stain for the acetylcholinesterase that is specific for rodent megakaryocytes. Such experimental systems, for the first time, have permitted more direct examination of the events that occur early in rodent megakaryocytopoiesis preceding those that have already been well studied in the morphologically recognizable megakaryocyte compartment.

Similar studies of human CFU-M have been delayed by the absence of an unambiguous megakaryocyte phenotypic probe with which to identify the megakaryocyte colonies cloned *in vitro*. Unfortunately, acetylcholinesterase is not contained in human megakaryocytes (30) and no analogous histochemical marker is known to exist. Previous studies of human CFU-M have relied exclusively upon morphologic criteria to delineate the cultured megakaryocytic colonies (1–3). We have recently described the utility of a rabbit antiserum directed against purified PGP as a phenotypic marker for the later cells of megakaryocytic lineage and have used this antisera in concert with indirect immunofluorescent staining to establish an assay for human peripheral blood CFU-M (4, 5). In this present report we extend our observations to human bone marrow CFU-M.

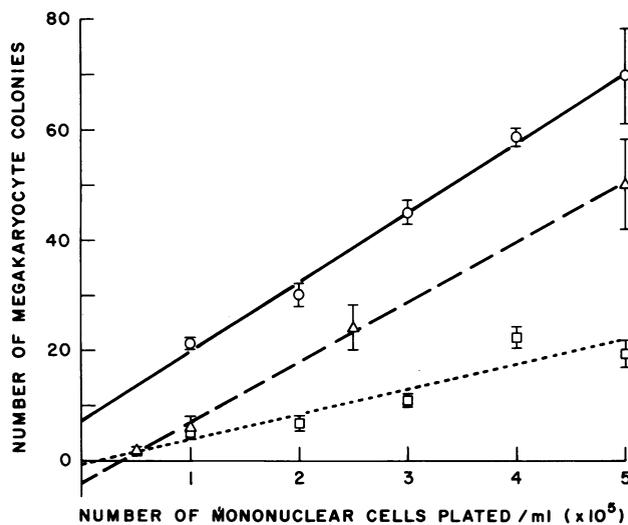


FIGURE 5 Effect of plated bone marrow mononuclear cell concentrations on the number of CFU-M-derived colonies cultured in the presence of 10% aplastic anemia serum. Each point is expressed as the mean  $\pm$  SEM of an experiment performed in duplicate to quadruplicate. The results of three separate experiments are shown and the data are fitted to least squares regression lines. In each experiment, the number of megakaryocyte colonies are linear with respect to the number of cells plated and the Y intercepts  $[y(0)]$  are approximately equal to zero.  $\circ$ :  $Y(0) = 7.15$ ,  $r = 0.972$ ,  $P < 0.01$ ;  $\triangle$ :  $Y(0) = -4.05$ ,  $r = 0.971$ ,  $P < 0.01$ ;  $\square$ :  $Y(0) = -0.63$ ,  $r = 0.854$ ,  $P < 0.01$ .

Bone marrow mononuclear cells that are cloned in our assay system in the absence of any megakaryocyte growth stimulator produce small numbers of megakaryocyte colonies, a finding similar to that which we have reported for peripheral blood (4, 5). It is unknown whether these colonies are the progeny of a subpopulation of CFU-M with an independent proliferative capacity, result from in vitro cellular interactions or products, or are supported by the low level of a trophic factor present in normal human sera. Similar growth of megakaryocyte colonies in the absence of a specific growth-promoting factor has been reported by others for both murine (31) and human (1-3) in vivo studies. The finding of unstimulated in vitro megakaryocytopoiesis is an observation consistent with animal studies in which marrow megakaryocytopoiesis persists despite intensive platelet hypertransfusion (32).

Our prior studies with peripheral blood CFU-M (4, 5) and those with bone marrow CFU-M reported here consistently demonstrate the failure of those substances active either in promoting granulocyte-macrophage or erythroid colony formation to be active in supporting CFU-M-derived colony growth. These substances include two sources of granulocyte-macrophage stimulating activity (E-CM and TC-CM), a source of erythroid burst-promoting activity (TC-CM) and partially purified erythropoietin. The failure of erythropoietin to increase CFU-M-derived colony growth in our system contrasts with the work of Vainchenker et al. (1-3) in man and that of McLeod et al. (6) in the mouse, but it is in agreement with the murine studies of Erslev et al. (31).

The only substance tested that consistently augments megakaryocyte colony formation is serum obtained from patients with severe aplastic anemia. The nature and specificity of this (Meg-CSA) are completely undefined. Serum and urine specimens from patients with aplastic anemia are known to have increased levels of erythropoietin (21, 22) and burst promoting activity (33, 34). Both normal and aplastic anemia human sera also have been reported to contain granulocyte-macrophage colony-stimulating activity (35-37). Others, in contrast, have reported consistent inhibition of CFU-GM colony formation by aplastic anemia sera (38).

We did not attempt to segregate the multiple activities of aplastic sera. Specificity of Meg-CSA, if it exists, is only suggested by the failure of the other known in vitro hematopoietic stem cell regulators to affect megakaryocyte colony formation.

An independent humoral regulator of thrombopoiesis has long been postulated (39). Careful studies in animals have reproducibly demonstrated the presence of thrombopoietin, a serum factor experimentally induced by acute thrombocytopenia that increases the incorporation of either  $^{75}\text{Se}$ -selenomethionine or  $^{35}\text{S}$ -

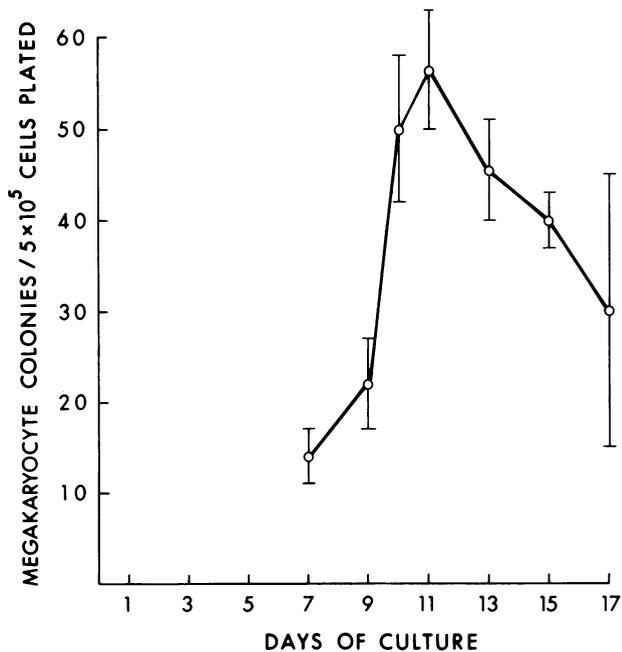


FIGURE 6 Number of CFU-M-derived megakaryocyte colonies assayed as a function of the number of days in culture. Results are expressed as the mean  $\pm$  SEM of a representative experiment performed in duplicate in the presence of 10% aplastic anemia serum. The original cell concentration plated was  $5 \times 10^5$  cells/ml.

sulfate into the platelets of thrombocytotic animals (32, 40, 41). Unfortunately, the physiologic significance of thrombopoietin is unknown because it does not reproducibly increase platelet counts in any experimental system. Studies in which human serum samples from patients with thrombocytopenia and thrombocytosis are assayed for thrombopoietin do not yield a pattern of findings for which a consistent regulatory hypothesis can be proposed (42). The relationship of Meg-CSA to thrombopoietin is unknown. Heterology is suggested by the failure of one source of thrombopoietin, HEKM (7), to increase megakaryocyte colonies in our assay system. Other investigators, however, have demonstrated the presence of megakaryocyte colony-stimulatory activity in HEKM (43, 44) using a murine assay system.

This study provides evidence that a humoral regulator of megakaryocytopoiesis, regardless of its specificity, does indeed exist and can be detected in sera from patients with aplastic anemia. Data similar to our own have recently been published by Enomoto et al. (45). In their study, urinary extracts from patients with aplastic anemia significantly stimulated both peripheral platelet counts and marrow megakaryocyte numbers in experimental animals. The extract was also active in enhancing rodent CFU-M colony formation in vitro (45). Conversely, however, Metcalf (46) found

that murine megakaryocyte colony formation was not stimulated by any of over 4,000 human sera from patients with a full range of hematologic disorders including aplastic anemia. Further studies will be necessary to define the biochemical identity of Meg-CSA, its specificity, presence, or absence in a variety of disease states, and its mechanism of action.

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