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### Research Article

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# In Vitro Studies of Human Pluripotential Hematopoietic Progenitors in Polycythemia Vera

## DIRECT EVIDENCE OF STEM CELL INVOLVEMENT

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**ABSTRACT** Previous in vitro studies on committed hematopoietic progenitors have suggested that polycythemia vera (PV) is a clonal disorder arising in a pluripotential hematopoietic stem cell. In this study, recently developed technics for clonal assay of a human multipotential progenitor cell (CFU-GEMM) were used to assess the functional characteristics of CFU-GEMM in 19 PV patients. These studies showed: (a) increased numbers of detectable CFU-GEMM in blood and bone marrow samples of PV patients as compared with normals ( $P < 0.002$  and  $P < 0.02$ , respectively); (b) erythropoietic differentiation of PV CFU-GEMM without exogenous erythropoietin (Ep) in culture (in marked contrast to CFU-GEMM of both normals and subjects with secondary erythrocytosis which require exogenous Ep for terminal hemoglobinization of their erythroid component), a property shown by experiments with an anti-Ep antiserum to be related to increased sensitivity of PV CFU-GEMM to Ep; (c) increased megakaryocyte formation by PV CFU-GEMM as compared with normals ( $P < 0.025$ ); and (d) a linear relationship, extrapolating to the origin, between CFU-GEMM detected and cells cultured. These studies demonstrate that at least two clinical features of PV, increased erythropoiesis and megakaryocytopoiesis, are reflected in corresponding functional characteristics of PV CFU-GEMM, and provide direct evidence of distinctive pluripotential stem cell activity in this disorder.

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## INTRODUCTION

Polycythemia vera (PV)<sup>1</sup> is a myeloproliferative disorder characterized by overproduction of erythrocytes, leukocytes, and platelets. The concept that this disorder originates in a pluripotential stem cell common to at least these three cell lineages was strongly advanced by studies of two PV G6PD heterozygotes with X-mosaicism, in whom circulating erythrocytes, granulocytes, and platelets were found to contain only a single type of G6PD isoenzyme, in contrast to normal tissue (skin fibroblasts and lymphocytes) which contained both isoenzyme types (1). Most in vitro studies providing support for the clonal nature of this disorder have used assay systems for various classes of committed hematopoietic progenitor cells. The in vitro growth of bone marrow cells of PV patients is notable for the presence of a population of erythroid precursor cells (endogenous colony forming units), not found in normals, with altered proliferative activity and sensitivity to very low levels of erythropoietin (Ep) (2, 3). These endogenous colony forming cells have a higher sedimentation velocity (4) and exhibit greater sensitivity to [<sup>3</sup>H]TdR suicide (5) than do normal erythroid progenitors. Moreover, although both normal and abnormal erythroid progenitors can be detected in PV,

<sup>1</sup> *Abbreviations used in this paper:* BFU-E burst forming unit-erythroid; CFU-E, colony forming unit-erythroid; CFU-C, granulocyte-macrophage colony forming unit; CFU-GEMM, colony forming unit giving rise to mixed colonies with granulocytic, erythroid, macrophage, and megakaryocytic elements; CFU-S, colony forming unit-spleen; CSF; granulocyte-macrophage colony stimulating factor; Ep, erythropoietin; FCS, fetal calf serum; PHA-LCM, media conditioned by human blood leukocytes in the presence of phytohemagglutinin; PV, polycythemia vera.

the abnormal population apparently gives rise to mature erythrocytes (6). Evidence has also been presented for a population of abnormal granulocyte-macrophage colony forming cells (CFU-C) with distinct sedimentation characteristics and sensitivity to [<sup>3</sup>H]TdR suicide, which, perhaps by a process of clonal suppression of normal CFU-C, account for the granulopoiesis in these patients (7).

The recent development of an *in vitro* assay for a progenitor cell (CFU-GEMM) that produces mixed colonies containing granulocytic, erythroid, macrophage, and megakaryocytic elements allows assessment of a human pluripotential hematopoietic stem cell (8, 9). This study examines the characteristics of the CFU-GEMM in PV, and provides direct evidence of involvement of the pluripotential myeloid stem cell in this clonal disorder.

## METHODS

**Blood and marrow samples.** Informed consent was obtained from all donors before obtaining samples. Heparinized blood and marrow aspirates were obtained from 15 normal adult donors and 19 patients with PV. All patients fulfilled diagnostic criteria for PV used by the National Polycythemia Vera Study Group (10). Two patients were studied at time of diagnosis; the remaining 17 were studied from 4 mo to 20 yr (median, 46 mo) from diagnosis. Median patient age was 65 yr (range 51–80 yr). Current or recent therapy at the time of study included: no treatment (two patients), phlebotomy alone (three patients), <sup>32</sup>P plus phlebotomy (nine patients), <sup>32</sup>P plus hydroxyurea (three patients), hydroxyurea plus phlebotomy (one patient), and pipobroman (one patient). Blood and marrow mononuclear cells were obtained by Ficoll-Hypaque gradient centrifugation (9). Three additional subjects with secondary erythrocytosis related to hypoxemic chronic lung disease (with elevated serum Ep levels) were also studied.

**Assay for CFU-GEMM and burst-forming unit-erythroid (BFU-E).** A modification (9) of the methyl cellulose assay for CFU-GEMM described by Fauser and Messner (8) was used. Serial concentrations of 0.25–4 × 10<sup>5</sup> marrow cells and 2–8 × 10<sup>5</sup> peripheral blood cells were cultured in the presence or absence of 1 IU Ep in quadruplicate 1-ml aliquots in 35 mm culture dishes containing 0.9% methyl cellulose, 5% PHA-LCM (media conditioned by human blood leukocytes in the presence of phytohemagglutinin), 30% fetal calf serum, Iscove's modified Dulbecco's medium, and 50 μM mercaptoethanol; Ep was added at the start of the culture. Plates were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> and high humidity, and colonies enumerated by direct observation *in situ* at ×30–200 on day 15. Hemoglobinized colonies, easily recognizable by their red color, consisted of those with pure erythroid elements (BFU-E) and mixed cellular elements (CFU-GEMM) in which an identifiable periphery of nonhemoglobinized small and large cells could be seen (9). Granulocyte-macrophage colonies (CFU-C derived) could also be recognized at this time as morphologically distinct colonies with a flatter arrangement of nonhemoglobinized cells. Individual mixed colonies were obtained by micropipetting, applied to glass slides, and stained with Wright-Giemsa for morphologic analysis (9). Selected colonies were also examined after myeloperoxidase, periodic

acid-Schiff, acid phosphatase, or nonspecific esterase staining to confirm their cytologic composition (9). The megakaryocyte content of the mixed (CFU-GEMM-derived) colonies has also been confirmed by immunofluorescent analyses using both a megakaryocyte-platelet selective heteroantiserum raised against a human platelet glycoprotein extract (11) (kindly provided by Dr. Ron Hoffman) and a monoclonal antibody, Tab, which recognizes a functionally specific glycoprotein complex (IIb-IIIa) of human megakaryocyte-platelet lineage (12, 13). To reduce the possibility that mixed colonies obtained for morphologic analysis might represent confluence or overlap of progeny of two or more committed progenitors seeded in close proximity in culture, colonies chosen for analysis were: (a) obtained only from plates of cells cultured in plating densities of ≤2 × 10<sup>5</sup>, (b) selected for analysis only when they appeared to be discrete single colonies in nonconfluent areas of the plates, and (c) lifted from plates in as little culture media (usually <2–3 μl) as possible during micropipetting. Similarly, colonies were scored as mixed for counting purposes only when they appeared to be discrete single colonies in nonconfluent areas of plates, so that all CFU-GEMM numbers used in this report were obtained from plates cultured at densities of ≤2 × 10<sup>5</sup> cells/ml for marrow samples and ≤4 × 10<sup>5</sup> cells/ml for blood samples.

**Assay for colony forming unit-erythroid (CFU-E).** The plasma clot system was used as previously described (2). Marrow samples were cultured at 2–6 × 10<sup>5</sup> cells/ml in quadruplicate 0.1-ml aliquots with and without 1.5 IU Ep, and benzidine-positive colonies were scored on day 7.

**Anti-Ep antibody studies.** Rabbit antiserum to human Ep was prepared, absorbed against pooled urinary protein of PV patients, and characterized as previously described (2). The antiserum used in these experiments neutralized ~300 IU Ep/ml undiluted, and a stock solution of this antiserum at a 1:10 dilution was used for addition to culture in those experiments described in Fig. 2. In these experiments (Fig. 2) serial dilutions of this 1:10 stock solution of anti-Ep antiserum were added in 100-μl aliquots to culture plates on day 0, and colonies were scored on day 15 as described above. In control experiments, similar dilutions of preimmune rabbit serum were added to culture plates. In additional studies, according to procedures previously detailed (2), fetal calf serum (FCS) used for culture was pretreated with either anti-Ep antiserum or nonimmune rabbit serum and subsequently incubated with goat anti-rabbit gamma globulin; the FCS so treated was then examined for its ability to support mixed colony formation. The Ep used in these studies was prepared by DEAE cellulose chromatography (14) from the urine of an anemic patient, and had a specific activity of 60 IU Ep/mg powder.

**Statistical considerations.** All culture data presented in this paper represent the mean ± SE of quadruplicate cultures at individual data points. *P* values have been calculated throughout by means of Student's *t* test.

## RESULTS

Figs. 1A and 1B show representative mixed colonies obtained from blood and marrow samples of PV patients cultured *in vitro* in the absence of added Ep. Fig. 1A shows a single colony comprising a dark central compact area of hemoglobinized cells and a surrounding "lawn" of nonhemoglobinized cells. Fig. 1B shows a similar colony containing numerous large cells, many

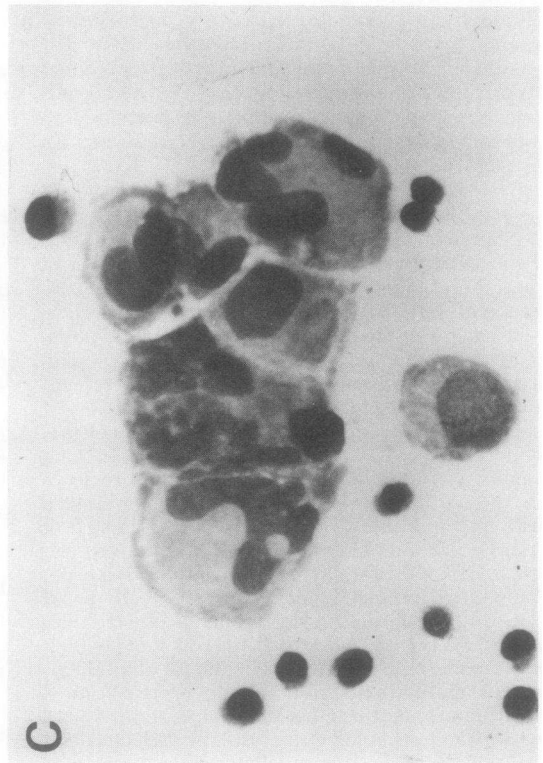
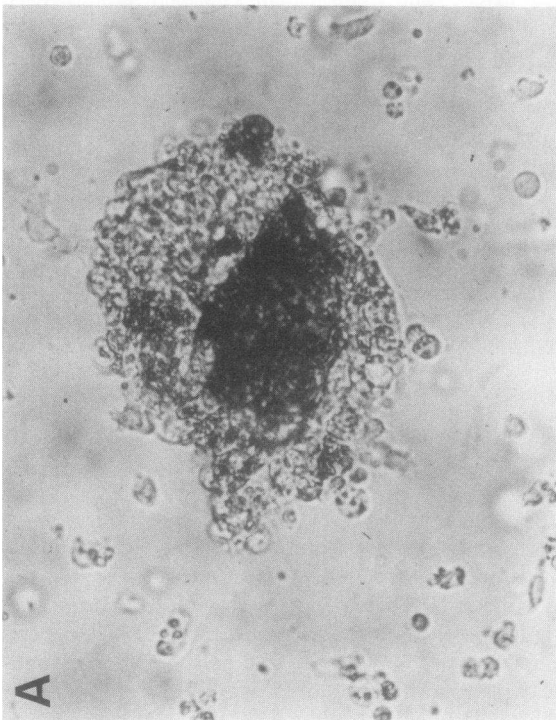
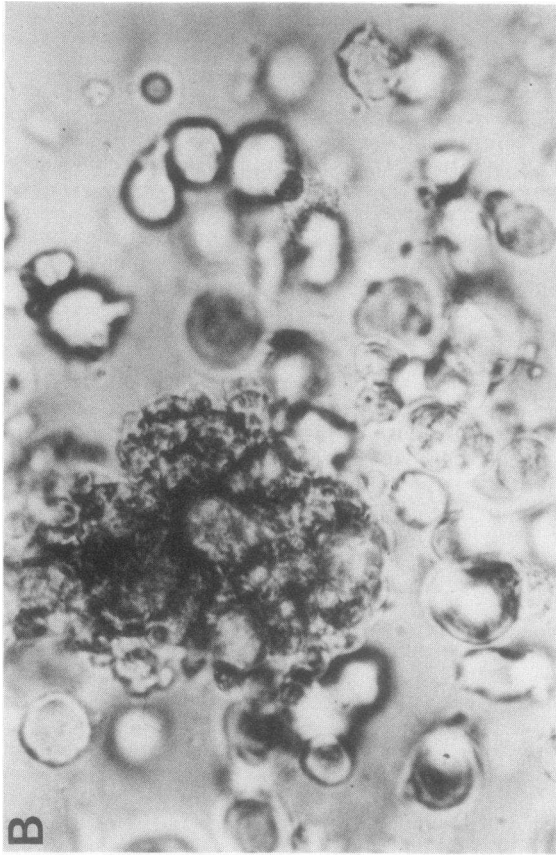


FIGURE 1 Endogenous mixed colonies in PV. A and B, representative colonies as they appear *in situ* on day 15 of culture (at  $\times 100$  and  $\times 200$ , respectively). C and D, selected photomicrographs of cells from individual PV mixed colonies after Wright-Giemsa staining, demonstrating a megakaryocyte cluster surrounded by small erythroblasts (C,  $\times 400$ ) and a single large megakaryocyte (D,  $\times 1,000$ ).

TABLE I  
Cellular Composition of CFU-GEMM-derived Mixed Colonies of Normal Donors and PV Patients

	Normal	PV
Number of mixed colonies examined*	231	163
Number of colonies containing:		
Granulocytic and erythroid cells only	60 (26%)†	32 (20%)
+ Macrophages	143 (62%)	114 (70%)
+ Eosinophils	9 (4%)	13 (8%)
+ Megakaryocytes	45 (19%)	72 (42%)
Number of megakaryocytes per colony‡		
Mean±SEM	5.5±2	22±8 <sup>  </sup>
Range	1-12	1-156

\* Combined results from eight normal donors and 10 PV patients.

† Number in parentheses indicate the percentage of total number of colonies.

‡ Only mixed colonies containing megakaryocytes were considered.

<sup>||</sup>  $P < 0.025$ , compared with normal.

of which were characterized by a hyaline and very translucent appearance in direct light microscopy *in situ*, and which were demonstrated in stained preparations of individual colonies to be megakaryocytes. Figs. 1C and 1D show selected areas of such Wright-Giemsa stained colonies: in Fig. 1C a megakaryocyte

cluster is surrounded by small erythroblasts; in Fig. 1D a higher-power view of a single megakaryocyte from a PV mixed colony is shown.

Table I compares the cellular composition of mixed colonies from blood and marrow of 10 PV patients with similar colonies of eight normal subjects cultured in the presence of Ep. These data demonstrate that, although all colonies contained at least granulocytic and erythroid elements and approximately two-thirds also contained macrophages, greater numbers of mixed colonies from PV patients (42 vs. 19%) contained megakaryocytic elements. Furthermore, when megakaryocyte-containing mixed colonies were scored for content of easily characterized megakaryocytes, PV patients contained significantly greater ( $P < 0.025$ ) numbers of megakaryocytes per colony.

Table II shows the number of mixed (CFU-GEMM) and erythroid (BFU-E and CFU-E) colonies obtained from blood and marrow samples of 19 PV patients and 12 normal subjects. The numbers of both mixed and pure erythroid colonies were moderately, but significantly, increased in PV patients as compared with normal subjects when samples were cultured in the presence of Ep. When samples were cultured without added Ep, however, all PV patients demonstrated both mixed and erythroid colony growth while normals showed no CFU-E and only rare and poorly hemoglobinized BFU-E or CFU-GEMM derived colonies (in 3 out of 15 subjects). The mean number of mixed colonies detected in PV blood samples cultured in the absence of Ep was ~45% (range 3-96%) of that detected when samples were cultured with added Ep.

Table II also shows results of similar cultures of

TABLE II  
Comparison of Mixed and Erythroid Colony Growth in PV and Normal Donors

		Blood		Bone marrow		
		Normals (n = 10)	PV (n = 19)	Normals (n = 15)	PV (n = 5)	<sup>2*</sup> Erythrocytosis (n = 3)
CFU-GEMM	+Ep	3.7±1.4	10.6±2.4†	12.8±3.9	41.2±7.2‡	12.4±3.4
	-Ep	≤0.1	6.4±1.8	≤0.05	20.6±8.8	≤0.05
BFU-E	+Ep	8.1±2.2	21.8±4.5 <sup>  </sup>	28.7±5.4	92.8±21 <sup>¶</sup>	33.6±6.8
	-Ep	≤0.5	12.9±3.4	≤0.05	46.4±12	≤0.05
CFU-E	+Ep	—	—	192±26	487±102	225±29
	-Ep	—	—	0	52±3.6	0

\* Each value represents mean±SEM of results from all donors in each group calculated to reflect the number of colonies/10<sup>5</sup> cells.

†  $P < 0.002$ , compared with normal blood.

‡  $P < 0.02$ , compared with normal marrow.

<sup>||</sup>  $P < 0.02$ , compared with normal blood.

<sup>¶</sup>  $P < 0.05$ , compared with normal marrow.

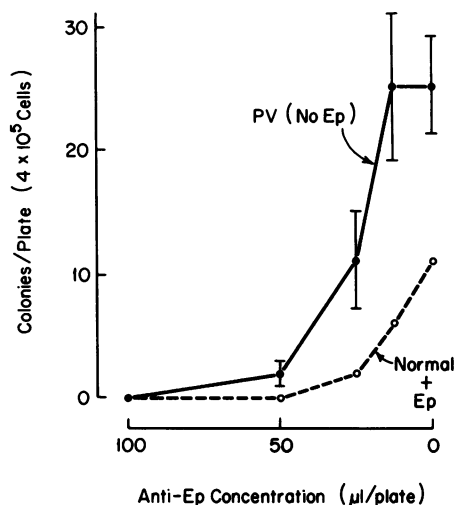


FIGURE 2 Effect of anti-Ep on mixed colony formation. The upper curve (●) shows mixed colony formation from blood of three PV patients (mean  $\pm$  SE) cultured without exogenous Ep, in the presence of decreasing concentrations of rabbit anti-Ep antiserum, beginning with 100  $\mu$ l of the 1:10 stock anti-Ep. The lower curve (○) shows comparable mixed colony formation from blood of a normal subject (cultured with 1 IU/ml exogenous Ep) in the presence of anti-Ep.

marrow from three subjects with secondary erythrocytosis. For these subjects the numbers of both mixed and erythroid colonies were similar to normals and, as in normals, no detectable erythroid or mixed colony formation was seen in cultures without added Ep.

The data shown in Fig. 2 demonstrate that the *in vitro* formation of recognizable CFU-GEMM-derived mixed colonies from blood samples of PV patients, cultured in the absence of Ep, can be prevented by the presence of an anti-Ep antiserum in a dose-dependent fashion. Mixed colony formation from a normal subject, cultured in the presence of exogenous Ep, is similarly inhibited (Fig. 2). In all such experiments BFU-E-derived erythroid colony formation was also inhibited by anti-Ep (data not shown). However, the formation of background numbers of granulocyte-macrophage colonies in these as well as normal cultures was not inhibited by the presence of anti-Ep, and growth of erythroid and mixed colonies could be restored by addition of excess Ep in cultures containing anti-Ep, which demonstrates that the anti-Ep serum was not nonspecifically toxic to progenitor cells in these culture conditions. The formation of megakaryocytes within CFU-GEMM-derived mixed colonies was not prevented by the presence of anti-Ep serum, and those mixed colonies observed in plates that contained subinhibitory concentrations of anti-Ep, or in which excess Ep was added to restore erythroid colony formation, were not morphologically different from those ob-

served when no anti-Ep was present. In those experiments from which the data shown in Fig. 2 were obtained, addition of equal concentrations of control preimmune rabbit serum did not result in inhibition of colony formation (data not shown).

In additional experiments the FCS used for culture was first pretreated with anti-Ep antiserum or control preimmune rabbit serum and subsequently incubated with goat anti-rabbit gamma globulin in order to remove Ep from FCS by immune precipitation before culturing. Those cultures of PV samples containing FCS treated with specific anti-Ep antiserum did not support mixed colony formation, whereas those using FCS treated with control preimmune serum grew expected numbers of mixed colonies, thus confirming the results obtained when anti-Ep antiserum was added directly as an Ep neutralizing agent in culture. For normal samples, FCS treated with either anti-Ep or control preimmune serum supported mixed colony formation only after the addition of exogenous Ep.

Fig. 3 shows data of a representative experiment from a PV subject that demonstrate that the number of CFU-GEMM-derived mixed colonies detected has a linear relationship, extrapolating to zero, to the number of cells plated. This relationship was seen for PV samples cultured with and without Ep and extended to low plating densities in all experiments involving three patient samples similarly studied, although at cell densities  $\leq$  50,000 cells/ml the assay efficiency was somewhat reduced.

## DISCUSSION

The CFU-GEMM assay utilized in this study allows assessment of a distinct class of human hematopoietic progenitor cells that exhibit at least some features similar to those that characterize the pluripotent stem cell of the mouse (CFU-S) (9). The biological significance of this assay for human multipotential progenitors has been underscored by analogous developments in *in vitro* culture for the murine model (15-19). In the murine system, cultures containing pokeweed mitogen-stimulated spleen-conditioned medium are now recognized to give rise not only to pure erythroid and granulocyte-macrophage colonies, but also to large complex colonies containing varying combinations of erythroid, granulocytic, macrophage, megakaryocytic, and eosinophilic elements (15-17, 19). In studies carried out by several investigators, the percentage of total erythroid burst colonies containing other hematopoietic elements has ranged from 30 to 50% (15, 16, 19). Evidence for a single cell, or clonal, origin of such colonies has been accumulated from cloning efficiency studies showing linearity to limiting plating dilutions (15, 16), recloning experiments transferring single-cell

suspensions of colonies at early stages of differentiation (15), and chromosomal marker analysis of individual mixed colonies formed in co-culture experiments (19). Studies have shown that the progenitor of at least the erythroid-megakaryocytic burst can undergo self-renewal during colony formation *in vitro* (17). Finally, both the ability of progenitors within individual macroscopic bursts to give rise to spleen colonies upon transfer into irradiated recipient mice (18) and the close correlation between the number of mixed colony progenitors and CFU-S in different hematopoietic tissues in the adult mouse (16) suggest that these colony-forming cells are members of the multipotential hematopoietic cell compartment with genuine stem cell properties.

Human CFU-GEMM are also functionally identified by their ability to form mixed colonies containing differentiated blood elements of several lineages. As demonstrated here and in previous reports (8, 9), such colonies are morphologically similar in their *in situ* appearance to those seen in murine culture studies (16). That human mixed colonies arise from single cells has been demonstrated for normal CFU-GEMM by Y chromatin analysis of individual mixed colonies formed in co-culture experiments involving male and female donor cells (8, 20), and has been supported by sedimentation velocity studies showing that these colony forming units have size characteristics of single cells similar to CFU-C and BFU-E (8). Recent improvements in culture technics (9, 21) have resulted in quantitative reproducibility and have clearly demonstrated that the assay plating efficiency for normal CFU-GEMM is linear with extrapolation to zero, which is also highly suggestive of single cell origin. Recloning experiments have shown that CFU-GEMM have a capacity both for giving rise to secondary colonies of differentiated committed progenitor type and for at least limited self-renewal *in vitro*, in that a proportion of such transfers give rise to secondary mixed colonies containing all lines of myeloid differentiation (9, 21). Finally, examination of the requirements for soluble factors necessary for *in vitro* growth provides evidence that CFU-GEMM are members of a distinct progenitor class and not simple overlap colonies (9). Cultures containing Ep and high granulocyte-macrophage colony stimulating factor (CSF) activity but without PHA-LCM produce large numbers of erythroid and granulocyte-macrophage colonies but no significant numbers of mixed colonies (9). This suggests the presence of an additional soluble hematopoietic factor in PHA-LCM necessary for CFU-GEMM growth and differentiation (9).

This study demonstrates that CFU-GEMM of PV patients manifest distinctive functional properties *in vitro* when compared with normals. Although each of

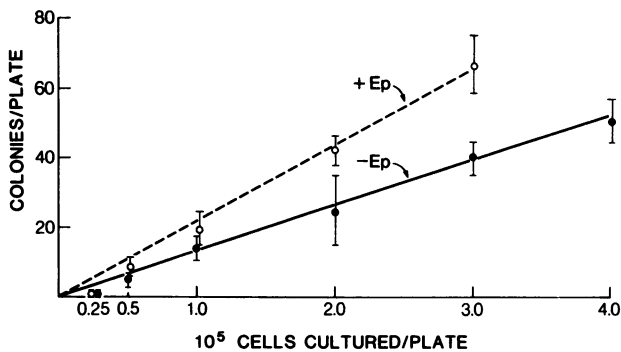


FIGURE 3 CFU-GEMM plating efficiency in PV. The data show a representative study of a PV patient, showing the linear relationship between number of marrow cells cultured, both with Ep (○) and without exogenous Ep (●), and the number of mixed colonies detected. Data shown are means  $\pm$  SE from four replicate cultures.

the experiments establishing clonality for normal CFU-GEMM-derived colonies has not been repeated, the linear relationship between the number of PV marrow cells plated and the number of mixed colonies formed provides strong evidence for the single-cell origin of such colonies, and indicates that the assay can be used quantitatively. Such pluripotential progenitors of PV patients can be detected *in vitro* in the absence of exogenous Ep, which suggests that the terminal hemoglobinization of the erythroid component within such mixed colonies has characteristics analogous to those previously demonstrated for the committed erythroid progenitors, the CFU-E (2-4) and BFU-E (6, 22) in PV. That formation of both recognizable mixed colonies and pure erythroid colonies can be prevented by anti-Ep suggests that erythropoietic differentiation and proliferation is not absolutely independent of Ep, but rather, that erythropoiesis by PV CFU-GEMM is sensitive to the low levels of Ep present in FCS, as has previously been demonstrated for the endogenous CFU-E (2). That such endogenous erythropoiesis was not seen for either pluripotential or committed erythroid progenitors from subjects with secondary erythrocytosis demonstrates that this phenomenon is not induced by a state of erythroid hyperplasia alone, and further suggests that this characteristic of PV progenitors may be of functional importance in the disordered erythropoietic regulation of the disease.

These experiments also demonstrate that *in vitro* megakaryocyte formation in colonies derived from PV CFU-GEMM is significantly increased when compared with normals, in whom the presence of small numbers of megakaryocytes in at least some mixed colonies had been previously demonstrated by selective staining and immunofluorescent techniques (9, 23). Limited



published data on [<sup>3</sup>H]TdR sensitivity suggest that PV CFU-GEMM may exist in an increased proliferative state compared to normals (24). Whether the increased megakaryocytopoiesis demonstrated here is dependent on cycle state characteristics of the PV CFU-GEMM or increased sensitivity to thrombopoietins present in the culture system is currently under investigation.

This study provides the first direct *in vitro* evidence that at least two clinical features of PV, augmented erythropoiesis and megakaryocytopoiesis, can be reflected in distinctive functional properties of the pluripotential progenitor cell compartment of such patients. The CFU-GEMM assay provides a useful tool for study of the regulation of hematopoietic stem cell activity in this disorder.

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