JCI The Journal of Clinical Investigation

Unregulated proliferation of primitive neoplastic progenitor cells in long-term polycythemia vera marrow cultures.

J D Cashman, … , C J Eaves, A C Eaves

J Clin Invest. 1988;81(1):87-91. https://doi.org/10.1172/JCI113315.

Research Article

Marrow cells from seven untreated patients with polycythemia vera (PV) were used to initiate standard single inoculum long-term marrow cultures. The numbers, erythropoietin independence, and cycling behavior of all detectable classes of erythroid, granulopoietic, and multilineage progenitors were then evaluated and the results obtained compared with preculture values. Time course studies showed that the long-term marrow culture system supports the continuous proliferation of primitive neoplastic progenitor cells from PV patients for many weeks. However, these progenitors fail to respond to signals from the adherent layer that return their counterparts in normal long-term marrow cultures to a quiescent state 5-7 d after each medium change. This abnormal cycling behavior of PV cells in the long-term culture system appears to mimic that operative in vivo, where primitive hemopoietic progenitors are also in a continuous state of turnover, in contrast to similar primitive progenitor compartments in normal individuals, which are quiescent. The long-term marrow culture system thus offers new possibilities for the further analysis of abnormal cellular and molecular mechanisms underlying clonal expansion at the stem cell level in PV.

Unregulated Proliferation of Primitive Neoplastic Progenitor Cells in Long-term Polycythemia Vera Marrow Cultures

J. D. Cashman, C. J. Eaves, and A. C. Eaves
The Terry Fox Laboratory, British Columbia Cancer Research Centre, Vancouver, British Columbia, Canada; and The Departments of Pathology, Medical Genetics, and Medicine, University of British Columbia, Vancouver, British Columbia, Canada

Abstract

Marrow cells from seven untreated patients with polycythemia vera (PV) were used to initiate standard single inoculum long-term marrow cultures. The numbers, erythropoietin independence, and cycling behavior of all detectable classes of erythroid, granulopoietic, and multilineage progenitors were then evaluated and the results obtained compared with preculture values. Time course studies showed that the long-term marrow culture system supports the continuous proliferation of primitive neoplastic progenitor cells from PV patients for many weeks. However, these progenitors fail to respond to signals from the adherent layer that return their counterparts in normal long-term marrow cultures to a quiescent state 5–7 d after each medium change. This abnormal cycling behavior of PV cells in the long-term culture system appears to mimic that operative in vivo, where primitive hemopoietic progenitors are also in a continuous state of turnover, in contrast to similar primitive progenitor compartments in normal individuals, which are quiescent. The long-term marrow culture system thus offers new possibilities for the further analysis of abnormal cellular and molecular mechanisms underlying clonal expansion at the stem cell level in PV.

Introduction

Polycythemia vera (PV)[1] is a myeloproliferative disorder characterized clinically by an increased red cell mass. Examination of marrow and blood cells from PV patients has shown that this clinical picture is secondary to the evolution in the patient of a single clone of self-renewing and differentiating pluripotent stem cells that expands abnormally (1). As a result, most of the circulating mature blood cells are part of the neoplastic clone, and the majority of primitive myeloid progenitors identified by clonogenic assays are neoplastic also (2). Kinetic studies of the most primitive progenitor compartments, which in normal individuals are largely quiescent (3–5), have revealed these to exhibit an accelerated turnover in patients with PV (6–8). Although evidence of this abnormality has not always been found (9), this may be explained by more recent studies showing that increased primitive progenitor cycling may be masked after prolonged storage of PV marrow or blood samples at 4°C and is reproducibly observed when samples are processed within 8 h of removal from the patient (Cashman, J. D., C. J. Eaves, and A. C. Eaves, unpublished observations). Nevertheless, there is no evidence that this readily detectable increased proliferation of primitive neoplastic cells results in a significant expansion in the size of any hemopoietic progenitor population in PV patients (10). The cause of the abnormal proliferative behavior of hemopoietic progenitor cells in PV is unknown. However, it seems possible that it might be linked at a genetic and molecular level to the same changes that confer upon cells at later stages of erythroid cell differentiation a decreased dependence on erythropoietin (Ep) receptor activation by specific ligand binding.

To pursue this question further, we sought a system where the abnormal proliferative behavior of primitive PV progenitors might be reproduced in vitro. Our previous studies had shown that populations of primitive hemopoietic progenitors that are normally quiescent in vivo undergo cyclic changes in their proliferative status in the adherent layer of long-term marrow cultures after each medium change (5). It has been reported that neoplastic progenitor populations can be maintained in long-term marrow cultures established from PV patients (11). We therefore set up a series of such cultures to evaluate the persistence and proliferative behavior of the neoplastic elements generated and maintained in this type of culture system. Our findings confirm the ability of such cultures to support neoplastic hemopoiesis from many PV patients and show that these cultures appear to mimic the lack of proliferation control characteristic of primitive PV progenitors present in vivo.

Methods

Patients. Marrow was obtained with informed consent as part of the diagnostic workup on all patients. Seven patients with a clinical diagnosis of PV were included in this study. All were untreated at the time of marrow aspiration. Marrow was collected in a small volume of serum-free tissue culture medium containing 800 U of heparin. Clinical and laboratory findings on each patient at the time of study are given in Table I. All patients showed Ep-independent erythroid colony and burst formation in standard methylcellulose assays (5–7, 12) of both blood and marrow. Marrow data are shown in Table I.

Long-term marrow cultures. Unprocessed marrow aspirate cells or marrow buffy coat cells were suspended in long-term marrow culture growth medium (αMEM plus 400 mg/liter glutamine, 40 mg/liter inositol, 10 mg/liter folic acid, 12.5% horse serum, 12.5% fetal calf serum, and 10^{-6} M hydrocortisone sodium succinate) to give a final concentration of $\sim 3 \times 10^6$ cells/ml as previously described (12, 13).

Address correspondence to Dr. Eaves, Terry Fox Laboratory, B. C. Cancer Research Centre, 601 W. 10th Ave., Vancouver, B. C. V5Z 1L3.

Received for publication 18 February 1987 and in revised form 17 August 1987.

1. *Abbreviations used in this paper:* BFU-E, erythroid burst-forming progenitors; CFU-E, erythroid colony-forming progenitors; CFU-GM, granulopoietic colony-forming progenitors; CML, chronic myeloid leukemia; Ep, erythropoietin; PV, polycythemia vera.

J. Clin. Invest.

0021-9738/88/01/0087/05 $2.00
Volume 81, January 1988, 87–91

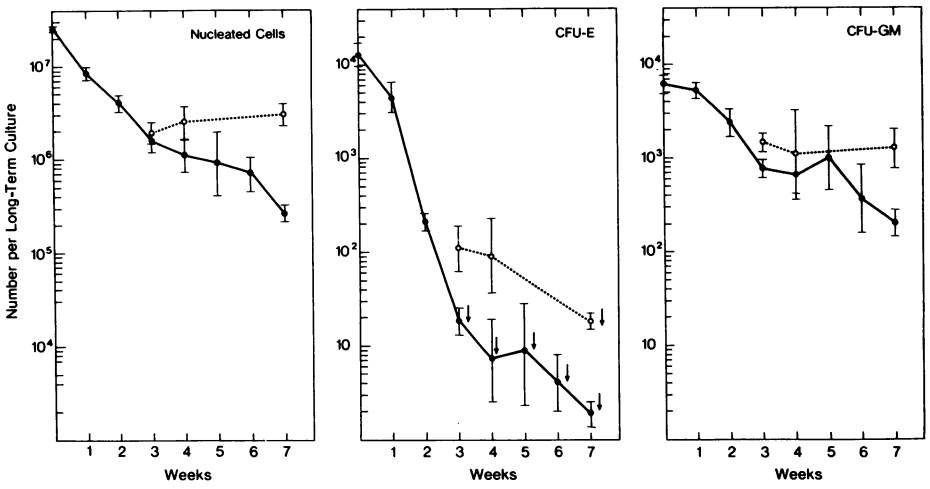

Figure 1. Total number of nucleated cells, CFU-E, and CFU-GM in the adherent (*open circles*) and nonadherent (*solid circles*) fractions of long-term PV marrow cultures assayed at varying times after initiation. Each point represents the geometric mean±1 SEM. The downward arrows indicate maximum mean values that would have been obtained if one colony had been seen in any of the assay dishes scored in an individual experiment.

8 ml (2.5×10^7 cells) were then placed in 60-mm dishes and the dishes incubated at 37°C for the first 3–5 d and at 33°C thereafter. Cultures were fed after the first 3–5 d by replacement of all the growth medium, and then again after each week by replacement of half the growth medium. Half of the nonadherent cells were also removed at each weekly feed and used to monitor the numbers of nucleated cells and granulopoietic (CFU-GM) and erythroid (CFU-E) colony-forming progenitors, and erythroid burst-forming progenitors (BFU-E) being maintained in the nonadherent fraction (12). To evaluate adherent layer progenitors, these cells were first suspended by exposure to collagenase, a procedure that then terminated the culture (12).

[^3H]Thymidine suicide technique. Washed marrow buffy coat or long-term culture cell suspensions, suitable for colony assay in methylcellulose (5–7, 12), were washed in nucleoside-free Hepes buffered medium, preincubated for 1 h at 37°C in nucleoside-free medium without serum, and then exposed for 20 min to 20 μCi/ml of high specific activity [^{3}H]thymidine (25 Ci/mmol) in a small volume at 37°C, pH 7.2, as previously described (5). Excess cold thymidine was then added and the cells washed and plated. A control aliquot was also always incubated in the same nucleoside-free medium without the addition of [^{3}H]thymidine and then plated in the same way. Clonogenic cells in S-phase during the 20 min of incubation with [^{3}H]-thymidine take up and incorporate a lethal dose of the nucleoside, resulting in their subsequent failure to form colonies. This suicide effect is represented as % kill = (control count − [^{3}H]thymidine count/control count) × 100%. Since approximately half of the cells in a totally cycling population will at any given time be in S-phase, a percent kill of > 10% is usually indicative of a significant cycling component. For cycling determinations (Table II), BFU-E and CFU-GM data are presented separately for progenitors of large and small colonies (i.e., to identify primitive and mature progenitor subpopulations, respectively) to allow comparisons with primitive cell types that in normal marrow are quiescent (3–5). For each determination shown, a minimum of 30 colonies of the relevant category were scored in control dishes (usually six replicate 1-ml methylcellulose assays, each containing 10^5 cells per milliliter) and an equivalent number of assay replicates were scored to obtain [^{3}H]thymidine-treated values.

Results

Cellularity and composition of PV long-term cultures. The total cellularity, as well as the CFU-E and CFU-GM progenitor content of both the nonadherent and adherent cell fractions monitored at weekly intervals, are shown in Fig. 1. BFU-E data are shown separately in Fig. 2. In general, the behavior of all of these cell populations in the seven long-term PV marrow cultures followed here was similar to that typical of long-term cultures initiated with marrow from normal individuals (12, 13). This pattern is characterized by an initial steep decline in the total nucleated cell and progenitor content of the nonadherent fraction for the first 3 wk of incubation during the formation of the adherent layer, after which an equilibrium is reached. During this second or "plateau" phase, which usually lasts for another 4–8 wk, adherent and nonadherent populations remain fairly constant, with the exception of CFU-E.

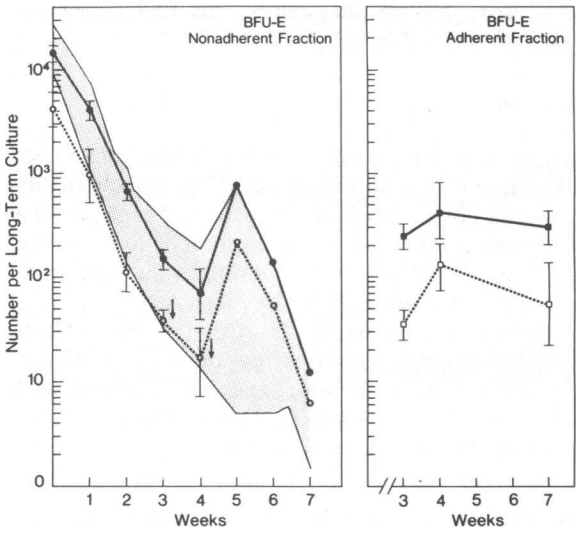

Figure 2. Changes in BFU-E numbers present in the nonadherent (*left*) and adherent (*right*) fractions of long-term PV marrow cultures as a function of time. The solid lines indicate the total number of BFU-E present in those cultures where the proportion of Ep-independent BFU-E (capable of burst formation in the absence of added Ep) was also assessed (*dashed lines*). For the nonadherent fraction such measurements were possible on all seven cultures up to week 4, but only on one culture thereafter. Assessment of the adherent layer required termination of a culture dish, and these measurements were therefore limited to three experiments at week 3, four at week 4, and three at week 7. Each point represents the geometric mean±1 SEM. The stippled area shows the range of total nonadherent BFU-E per long-term culture for all experiments (seven experiments up to week 4, and three thereafter).

These progenitors are typically not produced in significant numbers under regular long-term marrow conditions, and their numbers therefore decline rapidly to very low levels.

Ep independence in long-term PV cultures. The presence of neoplastic erythroid progenitors capable of forming colonies of mature, hemoglobin-containing progeny in methylcellulose assays in the absence of added Ep (< 0.002 U/ml) was determined intermittently for cells from both the adherent and nonadherent fractions of all seven experiments. A similar assessment was also made on the seven initial marrow specimens used to establish the cultures (Table I). The persistence of Ep-independent BFU-E in long-term PV cultures is shown in Fig. 2. The proportion of erythroid progenitors in both the adherent and nonadherent fractions that were capable of Ep-independent colony formation remained constant regardless of the age of the long-term culture and, in each experiment, this proportion was comparable with the corresponding value obtained in the initial assay of the marrow sample used to establish the culture. It would therefore appear that the abnormal clone showed no positive or negative selective advantage in the long-term PV marrow cultures in this study.

[^3H]Thymidine suicide assays of hemopoietic progenitors. The proliferative activity of primitive and mature hemopoietic progenitors in the adherent fraction of 3-, 4-, and 7-wk-old cultures was also determined. In the majority of experiments, additional dishes were killed 2 d after feeding for similar progenitor cell cycling determinations. Results are shown in Table II. Regardless of the age of the culture or the time since the previous medium change, the majority of cells in all classes of hemopoietic progenitor populations in the adherent layer appeared to be actively cycling, as reflected by the high suicide values they showed. For the primitive BFU-E and CFU-GM populations, this finding contrasts markedly with results obtained with normal marrow cultured using the same protocols and reagents and assessed in a similar fashion. In these, primitive BFU-E and CFU-GM in the adherent layer consistently show a low or undetectable S-phase component 7 d after the previous weekly feeding, and only after replacement of half of the culture medium are these transiently activated into S-phase (5).

In three experiments, a sufficient number of nonadherent cells were obtained to permit assessment of the proliferative state of progenitors in this fraction. As reported previously for normal marrow cultures, quiescent primitive BFU-E or mature CFU-M were not found in the nonadherent cell fraction of long-term PV marrow cultures at any time (5). Primitive CFU-GM were not detected in the nonadherent fraction of any cultures after 2 wk, and the number of nonadherent mature BFU-E detected was at no time sufficient to permit a meaningful determination of their cell cycle status.

Table I. Patient Data

Age/sex	Hg	WBC	Platelets	% Ep-independent*	
				CFU-E	BFU-E
	g%	*per mm³*	*per mm³*		
55/M	20.5	4,600	413,000	57 (129)	8 (133)
58/F	15.8	36,000	610,000	49 (84)	48 (171)
71/M	20.3	18,700	846,000	61 (97)	38 (56)
61/M	20.0	8,700	279,000	23 (131)	17 (136)
77/M	19.9	10,000	369,000	18 (100)	3 (68)
83/F	16.7	11,800	341,000	47 (181)	12 (35)
64/F	20.6	13,300	908,000	89 (335)	17 (97)

F, female; Hg, hemoglobin; M, male; WBC, white blood cells.
* Calculated by expressing the average number of erythroid colonies (CFU-E) or bursts (BFU-E) produced by 2×10^5 marrow buffy coat cells plated in methylcellulose assays (5–7, 12) without added Ep (< 0.002 U/ml) as a percent of the average number detected in simultaneously performed assays with 2×10^5 cells and optimal Ep (3 U/ml). Mean values for cultures with Ep are shown in parentheses.

Discussion

Studies of the cycling activity of clonogenic hemopoietic progenitors present in aspirates of marrow from normal individuals suggest that primitive but committed cell types are, like their pluripotent precursors, subject to mechanisms in vivo that establish a G_o population (3–5). It was also found that conditions prevailing in long-term marrow cultures allow these or similar mechanisms to operate in vitro (5), thus providing a system for their further analysis. In contrast, studies of hemopoietic progenitors taken directly from patients with PV have shown that analogous primitive populations in these individuals are in a state of rapid turnover (6, 7). This suggests that these cells may have an intrinsic abnormality that makes them insensitive to negative regulatory signals that render their normal counterparts quiescent.

To address this question, we established long-term cultures from PV marrow and investigated the Ep-independence and cycling behavior of the hemopoietic progenitors present several weeks later. In a previous study of the nonadherent fraction of such cultures, it was found that the proportion of Ep-independent erythroid progenitors remained unchanged over several weeks, suggesting that the majority of hemopoietic cells produced under these conditions remains clonal (11). Since we have found that the neoplastic clone usually disappears rapidly from long-term marrow cultures established from chronic myeloid leukemia (CML) patients (13), it was important to reexamine the persistence of Ep-independent erythroid progenitors in the present study and to include analyses of progenitors in the adherent layer, since > 80% of the more primitive types are normally located there (12). This distribution was found to be typical of long-term PV marrow cultures also and allowed us to confirm that Ep-independent erythroid progenitors continue to be maintained at the same level in the entire culture. It therefore seems likely that neoplastic stem cells are functional in standard long-term PV marrow cultures in contrast to the situation obtained with CML marrow. Although this difference remains unexplained, it does serve to highlight the biologic heterogeneity amongst different neoplastic clones associated with different myeloproliferative diseases.

The more significant observation reported here, however, is the continuous proliferative activity exhibited by primitive progenitors in the adherent layer of long-term PV marrow cultures. Negative regulation of analogous progenitor populations in normal long-term cultures appears to be a function of a nonhemopoietic cell population present in the adherent layer (14). The simplest explanation for the present findings is that neoplastic progenitors, which are able to bypass or ignore neg-

Table II. [^3H]Thymidine Suicide Values (Percent Kill) of Hemopoietic Progenitors in Long-Term PV Marrow Cultures

Cell fraction	Age of culture	Marrow source	Experiment no.	BFU-E*		CFU-GM‡	
				Primitive	Mature	Primitive	Mature
Adherent	3 or 4 wk	PV	1	55	57	—§	40
			2	32	47	30	61
			3	69	—	48	55
			4	50	54	53	54
			5	38	47	37	57
			6	58	47	57	55
			7	50	66	42	67
		Normal¶	$n = 16$	2±3	—	0±2	41±2
	3 or 4 wk	PV	3	52	65	58	49
	+ 2 d		4	42	71	53	46
			5	49	62	22	64
			6	62	50	54	52
			7	70	66	44	63
		Normal	$n = 15$	44±3	—	45±3	47±3
	7 wk	PV	5	43	—	11	57
			6	53	—	62	43
			7	53	64	48	51
		Normal	$n = 4$	3±2	—	10±3	52±7
	7 wk	PV	7	51	34	66	59
	+ 2 d	Normal	$n = 3$	33±4	—	43±4	45±4
Nonadherent	3 or 4 wk	PV	3	47	—	—	64
			4	45	—	—	52
			7	52	—	—	47
		Normal	$n = 6$	40±3	—	—	48±3
	4 wk	PV	7	56	—	—	62
	+ 2 d	Normal	$n = 3$	42±2	—	—	47±2

* Primitive, greater than eight clusters; mature, three to eight clusters. ‡ Primitive, > 500 cells; mature, 20 to 500 cells. § Insufficient numbers for cycling determination (i.e., < 30 colonies total in control assays. See Methods). ¶ Data from reference 5 and unpublished experiments.

ative regulatory signals from these adherent layer cells, represent the predominant phenotype in the most primitive compartments in both fresh and cultured PV marrow cell suspensions, and that the underlying defect is intrinsic to the primitive neoplastic PV cell. For example, an abnormality in the cell membrane of PV progenitors could prevent negative feedback interactions with regulatory cells. Alternatively, other abnormalities in gene expression could lead to an autocrine-like phenotype as has recently been suggested (15). Surface changes in malignant cells are also well known, and recently activation of hemopoietic growth factor gene expression (GM-CSF) in acute leukemia cells has been documented (16). The likelihood that there is a defect intrinsic to the neoplastic progenitor cell itself is strengthened by the results of studies of neoplastic progenitor cells from patients with CML. When the long-term marrow culture system is modified to allow the maintenance of primitive neoplastic CML progenitors in the presence of a normal marrow adherent layer, CML progenitors exhibit a similar lack of cycling control in vitro (14) as they also do in vivo (7, 17). However, neither our previous results with long-term CML cultures nor the findings reported here exclude the possibility of an indirect mechanism of neoplastic progenitor cell activation. To investigate whether the abnormal cycling control observed in long-term PV marrow cultures may be brought about indirectly by an abnormal regulatory population or by the inappropriate stimulation of normal regulatory cells, requires additional experiments to compare independently the interactions that occur in the adherent layer of long-term normal and PV cultures. Such experiments are in progress.

Acknowledgments

The secretarial assistance of M. Coulombe is gratefully acknowledged. The authors are also indebted to Dr. W. Boldt, Dr. G. Cantlie, Dr. K. Gelman, Dr. T. McMurtry, and Dr. R. Noble for providing patient specimens and relevant clinical data.

This work was supported by the National Cancer Institute of Canada, with core funding from the B. C. Cancer Foundation and the Cancer Control Agency of B. C. C. J. Eaves is a Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada.

References

1. Adamson, J. W., P. J. Fialkow, S. Murphy, J. F. Prchal, and L. Steinmann. 1976. Polycythemia vera: stem cell and probable clonal origin of the disease. *N. Engl. J. Med.* 295:913–916.

2. Prchal, J. F., J. W. Adamson, S. Murphy, L. Steinmann, and P. J. Fialkow. 1978. Polycythemia vera. The in vitro response of normal and abnormal stem cell lines to erythropoietin. *J. Clin. Invest.* 61:1044–1047.

3. Eaves, C. J., R. K. Humphries, and A. C. Eaves. 1979. In vitro characterization of erythroid precursor cells and the erythropoietic differentiation process. *In* Cellular and Molecular Regulation of Hemoglobin Switching. G. Stamatoyannopoulos and A. W. Nienhuis, editors. Grune & Stratton Inc., Orlando, FL. 251–278.

4. Fauser, A. A., and H. A. Messner. 1979. Proliferative state of human pluripotent hemopoietic progenitors (CFU-GEMM) in normal individuals and under regenerative conditions after bone marrow transplantation. *Blood.* 54:1197–1200.

5. Cashman, J., A. C. Eaves, and C. J. Eaves. 1985. Regulated proliferation of primitive hematopoietic progenitor cells in long-term human marrow cultures. *Blood.* 66:1002–1005.

6. Fauser, A. A., and H. A. Messner. 1981. Pluripotent hemopoietic progenitors CFU-GEMM in polycythemia vera: analysis of erythropoietin requirement and proliferative activity. *Blood.* 58:1224–1227.

7. Eaves, A. C., and C. J. Eaves. 1984. Erythropoiesis in culture. *In* Cell Culture Techniques: Clinics in Haematology. Vol. 13, No. 2. E. A. McCulloch, editor. W. B. Saunders Co. Ltd., Eastborne, England. 371–391.

8. Mladenovic, J., and J. W. Adamson. 1982. Characteristics of circulating erythroid colony-forming cells in normal and polycythemic man. *Br. J. Haematol.* 51:377–384.

9. Singer, J. W., P. J. Fialkow, J. W. Adamson, L. Steinmann, C. Ernst, S. Murphy, and K. J. Kopecky. 1979. Polycythemia vera: increased expression of normal committed granulocytic stem cells in vitro after exposure of marrow to tritiated thymidine. *J. Clin. Invest.* 64:1320–1324.

10. Eaves, A. C., and C. J. Eaves. 1983. In vitro studies of erythropoiesis in polycythemia vera. *In* Current Concepts in Erythropoiesis. C. D. R. Dunn, editor. John Wiley & Sons, New York. 167–187.

11. Powell, J. S., P. J. Fialkow, and J. W. Adamson. 1982. Polycythemia vera: studies of hemopoiesis in continuous long-term culture of human marrow. *J. Cell. Physiol.* 1:79–85.

12. Coulombel, L., A. C. Eaves, and C. J. Eaves. 1983. Enzymatic treatment of long-term human marrow cultures reveals the preferential location of primitive hemopoietic progenitors in the adherent layer. *Blood.* 62:291–297.

13. Coulombel, L., C. J. Eaves, I. D. Dubé, D. K. Kalousek, and A. C. Eaves. 1984. Variable persistence of leukemia progenitor cells in long-term CML and AML marrow cultures. *In* Long-Term Bone Marrow Culture. D. G. Wright and J. S. Greenberger, editors. Alan R. Liss, Inc., New York. 243–254.

14. Eaves, A. C., J. D. Cashman, L. A. Gaboury, D. K. Kalousek, and C. J. Eaves. 1986. Unregulated proliferation of primitive chronic myeloid leukemia progenitors in the presence of normal marrow adherent cells. *Proc. Natl. Acad. Sci. USA.* 83:5306–5310.

15. Eid, J., R. F. Ebert, M. S. Gesell, and J. L. Spivak. 1987. Intracellular growth factors in polycythemia vera and other myeloproliferative disorders. *Proc. Natl. Acad. Sci. USA.* 84:532–536.

16. Young, D. C., and J. D. Griffin. 1986. Autocrine secretion of GM-CSF in acute myeloblastic leukemia. *Blood.* 68:1178–1181.

17. Lepine, J., and H. A. Messner. 1983. Pluripotent hemopoietic progenitors (CFU-GEMM) in chronic myelogenous leukemia. *Int. J. Cell Cloning.* 1:230–239.