Chronic Myelogenous Leukemia

IN VITRO STUDIES OF HEMATOPOIETIC REGULATION IN A PATIENT UNDERGOING INTENSIVE CHEMOTHERAPY

JACK W. SINGER, JOHN W. ADAMSON, ZALMEN A. ARLIN, SANFORD J. KEMPIN, BAYARD D. CLARKSON, and PHILIP J. FIALKOW, *The Sections of Oncology, Hematology, and Medical Genetics, Medical Service, Veterans Administration Medical Center, Seattle, Washington 98108; Department of Medicine, University of Washington, Seattle, Washington 98195; Hematology-Lymphoma Service, Memorial Sloan-Kettering Cancer Center, New York 10021*

ABSTRACT A patient heterozygous for the X-linked enzyme glucose-6-phosphate dehydrogenase and with Philadelphia chromosome-positive chronic myelogenous leukemia (CML) was treated with combination chemotherapy and had a partial loss of Philadelphia chromosome accompanied by partial restoration of nonclonal hematopoiesis as determined by glucose-6-phosphate dehydrogenase. Studies of in vitro hematopoiesis were performed after chemotherapy to evaluate the influences of neoplastic stem cells on normal cells and to determine whether there were physical and cell kinetic differences between leukemic stem cells and their normal counterparts. The data revealed the following: (*a*) The frequencies of normal committed granulocytic stem cells (CFU-C) and erythroid stem cells (BFU-E) in blood did not differ from the frequencies in marrow. (*b*) Normal late erythroid progenitors (CFU-E) were found at a significantly lower frequency than the more primitive BFU-E. Calculations indicated that not only was there a decrease in CFU-E production by normal BFU-E, but there was also abnormal clonal expansion of CML BFU-E (CFU-E:BFU-E ratio for normal progenitors was 1.1, whereas for the CML clone it was 11.5). (*c*) No increase in frequency of normal CFU-C was found after marrow cells were exposed to high specific activity tritiated thymidine. (*d*) Normal CFU-C and those from the CML clone were not separable on the basis of density. (*e*) The frequency of normal BFU-E was consistently greater than that of CFU-C, suggesting that regulatory differences influence the commitment of normal progenitors to the two pathways.

Received for publication 29 September 1980 and in revised form 12 January 1981.

INTRODUCTION

Previous studies have shown that Philadelphia chromosome (Ph^1)[1]-positive chronic myelogenous leukemia (CML) is a clonal disease arising in a pluripotent stem cell (1–3). These conclusions were based on studies of 18 patients who had naturally occurring cellular mosaicism at the glucose-6-phosphate dehydrogenase (G-6-PD) locus. This system can be used as a marker for these studies because the structural gene for G-6-PD is on the X chromosome and undergoes inactivation early in embryogenesis. Thus, in a female heterozygous for G-6-PD, only one of two enzymes is synthesized in each cell; consequently normal tissues are mixtures of cells, some synthesizing type B and others, the variant, type A G-6-PD. In contrast, in a neoplasm that arises from a single cell, all malignant cells will have a single-enzyme type.

In a G-6-PD heterozygote with CML, intensive combination chemotherapy resulted in a partial loss of Ph^1 and a return of a double-enzyme G-6-PD phenotype in her myeloid cells (4). This report details studies of in vitro hematopoiesis on samples obtained from this patient after recovery from the first, second, and fourth courses of chemotherapy. Using colony growth in vitro to amplify the gene products of single progenitors, it is possible to infer the G-6-PD type of individual committed stem cells and thus to determine whether they are neoplastic or normal. Therefore, the influences of neoplastic stem cells on normal stem cells, and physical

[1] *Abbreviations used in this paper*: BFU-E, primitive erythroid stem cells; CFU-C, committed granulocytic stem cells; CFU-E, late erythroid progenitors; CML, chronic myelogenous leukemia; G-6-PD, glucose-6-phosphate dehydrogenase; Ph^1, Philadelphia chromosome.

and cell kinetic differences between the two types of cells, can be investigated.

In this study the following questions were addressed: (*a*) Are the frequencies of normal committed granulocytic (CFU-C) and erythrocytic (BFU-E) stem cells similar? (*b*) Are the frequencies of these normal progenitors similar in blood and marrow? (*c*) Are the frequencies of normal, late, and early erythroid precursor cells (CFU-E and BFU-E, respectively) similar? (*d*) Are the cell cycle characteristics of progenitors derived from the CML clone different from those of their normal counterparts? (*e*) Are normal progenitors physically separable from those of the CML clone?

METHODS

Subject. Clinical details of the patient's course were reported previously (4). Briefly, in March 1979, A.J., a 33-yr-old black female, was diagnosed to have Ph^1-positive CML. Hematologic values at that and subsequent times are shown in Table I. After informed consent was obtained, the first cycle of chemotherapy on the L-15 protocol was begun on 5/8/79. The chemotherapy consisted of daunorubicin (45 mg/m^2 i.v. daily for 2 d), cytosine arabinoside (12.5 mg/m^2 i.v., followed by continuous infusion at 100 mg/m^2 daily for 4 d), and 6-thioguanine (100 mg/m^2 q 12 h for 4 d). Three subsequent courses of the same chemotherapy were administered: 6/15/79, 7/23/79, and 8/20/79.

Marrow and peripheral blood samples obtained in New York on the dates given in Table I were drawn into preservative-free heparin (10 U/ml) and shipped by air express to Seattle, Wash. A full-thickness skin biopsy obtained from the patient was tested directly for G-6-PD by published methods (1). The 10/4/79 sample was obtained in Seattle at the time marrow was harvested for cryopreservation and later autologous transplantation.

Cell culture methods. Light-density peripheral blood or marrow mononuclear cells or both were separated on Ficoll-diazatroate gradients (Teva Limited, Jerusalem, Israel) and cultured for granulocytic and erythroid colony growth. Erythroid bursts were grown in the presence of 2.5 U of sheep plasma erythropoietin (step 3; Connaught Medical Research Laboratory, Willowdale, Ontario, Canada) as described (5). Erythroid colonies derived from CFU-E were counted and harvested for G-6-PD after 7 d of culture. At day 10–12 of culture, the number of bursts and their G-6-PD types were determined. Granulocytic colonies were grown with phytohemagglutinin-conditioned medium as a source of colony-stimulating factor, and were harvested and tested for G-6-PD after 12–14 d of culture (6).

All colonies that could be seen under a dissecting microscopy at ×40 in the culture dishes were plucked with a fine pipette and electrophoresed to determine their G-6-PD isoenzyme type. If growth was excessive, then all colonies in a single quadrant of the culture dish were analyzed. Approximately 90% of granulocytic colonies and erythroid bursts were analyzable for G-6-PD; however, only 30% of CFU-E produced readable bands. This reflects both their smaller colony size and the lesser amount of enzyme per cell.

Studies with tritiated thymidine ([^{3}H]TdR). Marrow cells in thymidine-free Eagle's medium (Microbiological Associates, Walkersville, Md.) were exposed for 20 min at 37°C to either high specific activity [^{3}H]TdR (New England Nuclear, Boston, Mass.) or to an equivalent amount of unlabeled thymidine (7). Cells were then washed twice with cold Eagle's minimal essential medium, containing 10% fetal calf serum (Reheis Co. Inc., Kankakee, Ill.) and 100 μg/ml of thymidine, recounted, and plated for colony growth.

Cell separation on discontinuous density gradients. Mononuclear cells were separated over a 10-step discontinuous density gradient made with Percoll (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) as previously reported (8). Approximately 5×10^8 cells were suspended in 1 ml of Dulbecco's phosphate-buffered saline (Gibco, Grand Island Biological Co., Grand Island, N. Y.) and layered onto the top of the gradient. The gradient was centrifuged at 350 *g* for 20 min at room temperature. Cell bands were harvested from the top in ~1-ml fractions which corresponded to stepwise increases in density between 1.050 and 1.090 g/ml. The cells in each fraction were washed once in 10 vol of Dulbecco's phosphate-buffered saline, resuspended in Eagle's medium, counted in a hemocytometer, and then plated for colony growth. Each fraction was also directly tested for G-6 PD type.

RESULTS

Before chemotherapy, the patient's erythrocytes, granulocytes, and platelets manifested only B-type G-6-PD activity. As shown in Table I, after each cycle of therapy, the frequency of Ph^1-negative marrow cells increased concomitantly with an increase in the percentage of A-type G-6-PD activity in the circulating granulocytes. Before chemotherapy, only 2 of 59 erythroid bursts and 0 of 50 granulocytic colonies showed type A enzyme activity. However, after the first cycle of chemotherapy, type A activity was detected in the circulating peripheral blood cells and in cultured colonies (Table II). After subsequent cycles of therapy, the frequency of type A colonies increased. On 10/4/79, 6 wk after the fourth cycle of chemotherapy, 50% of the erythroid bursts and 35% of the granulocytic colonies were type A.

The ratios of A:B G-6-PD in skin and blood are highly correlated. Thus, when approximately equal amounts of A and B enzyme activity are found in the skin, the percentage of normal colonies is assumed to be double the percentage of type A colonies in a G-6-PD heterozygote with a B-type clone. For every presumed normal A-type colony detected, one of the B-type colonies is also normal. Therefore, on 10/4/79, 100% of the erythroid bursts and 71% of the granulocytic colonies were derived from normal progenitors.

Table II shows the number and G-6-PD types of marrow-derived CFU-C, CFU-E, and BFU-E cultured on four separate occasions.

Frequency of normal CFU-C vs. BFU-E. The frequencies of types A and B CFU-C and BFU-E cultured simultaneously on three separate occasions are shown in Table II. Only on the specimen obtained on 1/24/80 were sufficient numbers of colonies harvested to adequately compare the frequencies of normal and CML CFU-C and BFU-E. On that occasion BFU-E were 86% normal, whereas CFU-C were only 22% normal ($P < 0.0025$; χ^2). In addition, a highly significant differ-

TABLE I
Clinical Data

Date	Prior therapy	Hemoglobin	Leukocyte count	Platelet count	Marrow cells with Ph¹	Normal granulocytes by G-6-PD
		g/100 ml	*mm³ × 10³*	*mm³ × 10³*	%	%
4/4/79	None	11.7	30.7	487	100 (31/31)*	0
6/5/79	Cycle 1 (5/18/79)‡				92 (92/100)	10–20
7/3/79	Cycle 2 (6/15/79)‡	12.5	6.8	490	43 (29/67)	80
10/4/79	Cycle 4 (8/20/79)‡				17 (12/50)	80
1/24/80	Cycle 4 (8/20/79)‡	15.1	7.0	237		40

* Number of marrow metaphases with Ph¹/number of metaphases analyzed.
‡ Starting date of most recent chemotherapy.

ence between the ratio of normal and CML BFU-E and CFU-C was obtained with a paired *t* test considering all samples ($P < 0.001$).

Erythroid progenitors. Table II gives the frequencies of normal CFU-E and BFU-E determined on three occasions. The apparent differences in frequencies of normal CFU-E and BFU-E were not significant for any one specimen due to small sample sizes. However, when the differences in frequency between normal BFU-E and CFU-E were compared for all three studies, the frequency of normal CFU-E was significantly less than normal BFU-E ($P < 0.05$; paired *t* test).

Studies with [³H]TdR. On the three occasions when marrow cells were exposed to [³H]TdR, the percentages of CFU-C actively synthesizing DNA were 12, 32, and 32%, values within or below normal values from this laboratory (31±7; mean±SD; $n = 16$; Table III). When the percent losses of clonal and normal colonies were calculated by applying the calculated percentages of normal and CML clonal colonies to the absolute number of colonies detected, the percentage of CFU-C in DNA synthesis from the CML clone appeared lower than that of the presumably normal CFU-C ($P < 0.1$; Student's *t* test).

Cell separation studies. When peripheral blood mononuclear cells were separated by a discontinuous density gradient on the specimen from 5/8/79 before the patient received chemotherapy, an increase in low density CFU-C was found (Fig. 1). After each cycle of therapy, CFU-C with a density of 1.065 g/ml increased; however, the modal density remained 1.060 g/ml. Peripheral blood CFU-C from normal donors have modal densities of 1.065—1.070 g/ml (8).When colonies were harvested from the gradients and tested for G-6-PD, the frequencies of A-type colonies were similar in the light and normal density regions (Table IV). Thus, CML clonal CFU-C and presumably normal CFU-C were not physically separable. Both had abnormally light buoyant densities.

DISCUSSION

In this study, we used the naturally occurring cellular mosaicism for G-6-PD to assess regulatory mechanisms

TABLE II
In Vitro Hematopoietic Colony Growth

			Granulocyte-macrophage colonies				Erythroid colonies									
				G-6-PD type				G-6-PD type				G-6-PD type			CFU-E:BFU-E ratios	
Date	Weeks after chemotherapy	Percent normal granulocytes	Colonies/ 10^5 cells ±SD	A	B	Percent normal	CFU-E colonies/ 10^5 cells	A	B	Percent normal	BFU-E colonies/ 10^5 cells	A	B	Percent normal	normal	CML clone*
6/5/79	3	10–20	80±12	2	15	24	—	—	—	—	—	—	—	—	—	—
7/3/79	3	80	43±8	2	12	29	85±5	2	13	27	28±5	14	56	40	2.0	3.7
10/4/79	6	80	45±4	11	20	71	19±2	3	7	60	21±7	14	13	100	0.5	>7.6‡
1/24/80	21	40	153±20	6	39	22	31±7	2	10	33	13±2	18	24	86	0.9	10.9

* The normal and CML-clonal CFU-E:BFU-E ratios were calculated by applying the estimated percentages of normal CFU-E and BFU-E to the absolute number of colonies detected.
‡ Since no CML clonal BFU-E were detected by G-6-PD on this date, this calculation is based on the assumption that the frequency of BFU-E derived from the CML clone was <1 of the 27 BFU-E analyzed for G-6-PD.

TABLE III
Effect of [³H]TdR on Granulocyte-Macrophage Colonies

Date	Colonies/10^5 cells±SD			Normal by G-6-PD		Loss	
	Control	[³H]TdR	Loss	Control	[³H]TdR	CML clonal	Normal
			%	%		%	
6/5/79	161±18	140±23	12	(2/15)* 24	(4/41) 18	7	33
7/3/79	97±17	65±6	32	(2/12) 29	(5/32) 27	32	36
10/4/79	90±7	61±3	32	(11/20) 71	(9/21) 60	12	42

* Numbers in parentheses represent the number of G-6-PD type A colonies:type B colonies.

in CML. Initial studies in 18 patients demonstrated that the disease was clonal and that it originated in a progenitor common to erythrocytes, platelets, and granulocytes (1–3, 9). Moreover, in five patients studied, only 2 of > 1,300 granulocytic colonies tested had the enzyme type different from that found in the CML clone, suggesting that significant numbers of normal multipotent stem cells might not be present in CML (10). However, when the present patient received intensive combination chemotherapy, nonclonal hematopoiesis, as determined by G-6-PD, was restored (4). This led to the conclusion that in CML, as in polycythemia vera, residual normal stem cells were present, but in CML their in vitro as well as their in vivo expression was suppressed. Examinations of several potential mechanisms for this suppression are detailed in this report.

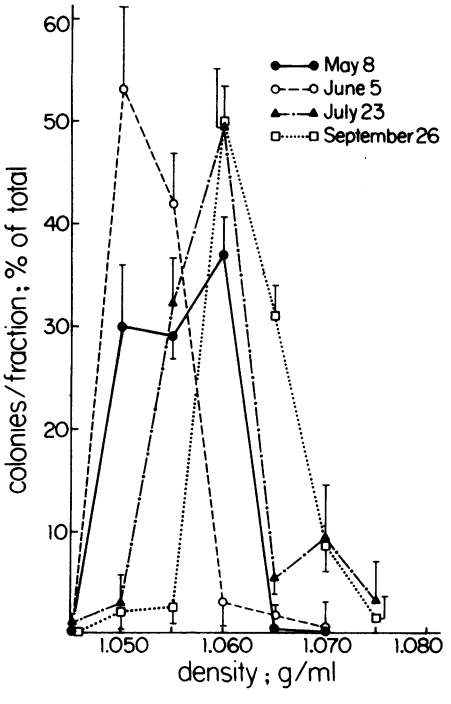

FIGURE 1 The CFU-C density profiles of four peripheral blood samples separated on discontinuous bouyant density Percoll gradients. The values represent the percent ±1 SD found in each fraction of the total CFU-C harvested from the gradient.

Studies of two G-6-PD heterozygotes with polycythemia vera demonstrated that circulating and marrow BFU-E grown with high doses of erythropoietin and CFU-C grown conventionally had approximately equal frequencies of normal colonies (19 and 18% normal BFU-E in marrow and peripheral blood, respectively, and 16 and 21% normal CFU-C in marrow and peripheral blood, respectively) (11). Although in the CML patient, after treatment the frequencies of normal progenitors were similar in marrow and peripheral

TABLE IV
Discontinuous Density Gradient Data

Date	Density of CFU-C	G-6-PD types		Percent normal
		A	B	
	g/ml			
3/29/79	<1.065	1	58	<5
	≥1.065	1	57	<5
6/4/79	<1.065	2	29	13
	≥1.065	3	21	25
7/23/79	<1.065	2	18	20
	≥1.065	0	4	<20
9/26/79	<1.065	19	41	63
	≥1.065	19	28	81

blood, the frequency of normal BFU-E was greater than the frequency of normal CFU-C (Table II). On 1/24/80, ~5 mo after completion of chemotherapy, 22% of the CFU-C were normal, whereas 86% of the BFU-E were normal ($P < 0.0025$; χ^2). This difference in in vitro expression of these two types of normal progenitors suggests that the relative rates at which restriction to the erythroid or granulopoietic pathways occurs in CML differs from normal and also differs from that found in polycythemia vera.

In the hierarchy of erythropoietic differentiation, BFU-E are thought to be closely related to multipotent stem cells and to give rise to the more mature CFU-E. In polycythemia vera, although normal BFU-E were detectable, a negligible number of normal CFU-E were detected in vitro or expressed in vivo, suggesting that normal BFU-E were suppressed from further in vivo maturation (11). In the CML patient, normal CFU-E were expressed with a frequency approximating that of normal circulating erythrocytes and with a lesser frequency than that of normal BFU-E (Table II). The CFU-E:BFU-E ratios of normal and clonal progenitors differed significantly as a result of two factors: an increase in the number of CFU-E produced by neoplastic progenitors and a decrease in those produced by normal BFU-E. The mean calculated CFU-E:BFU-E ratio for the CML clone was 11.5±4.0 (mean±SD) for the three studies, whereas for normal progenitors it was 1.1±0.8 ($P < 0.05$; paired t test). The ratio of CFU-E:BFU-E from 39 normal marrows was 2.8±1.2) (10). This difference in in vitro expression of normal and CML clonal erythroid progenitors is unlikely to have been due to nonsteady-state conditions because the 1/24/80 specimen was obtained 5 mo after the last chemotherapy.

The data from the [^{3}H]TdR studies indicate that normal granulopoiesis in vitro is regulated differently in CML than in polycythemia vera. Exposure of polycythemia vera marrow cells to [^{3}H]TdR resulted in significant increases in expression of normal CFU-C (7). In contrast, no such increase was noted in CML. In fact, on three occasions, normal CFU-C appeared to be dividing somewhat more rapidly than the neoplastic cells. Thus there was a net reduction in the frequency of normal CFU-C after exposure to [^{3}H]TdR. These data are also consistent with the postulate that in CML the regulatory abnormalities inhibiting the expression of normal progenitors involve stem cells less differentiated than detectable CFU-C or reside in an increased capacity for clonal expansion of CML committed progenitors.

In one patient with polycythemia vera it was possible to separate normal from clonal CFU-C by use of discontinuous density gradients and unit gravity velocity sedimentation (8). In contrast, in the CML patient no separation of normal from neoplastic CFU-C was observed (Table IV). The abnormally light density of apparently normal CFU-C suggests that these cells, although not derived from the dominant CML clone, might be abnormal. It is not likely that chemotherapy decreases the density of normal CFU-C because three patients with acute nonlymphocytic leukemia who received chemotherapy similar to that given to the CML patient had normal modal densities for CFU-C after entering remission (1.065, 1.065, and 1.070 g/ml (unpublished data).

The present results also indicate that the previously reported leukemic inhibitory activity (12, 13) of CML cells did not play a significant role in inhibiting the maturation of normal CFU-C in this patient after combination chemotherapy. However, such inhibitors could act on cells earlier in the differentiation scheme than those readily detectable by current in vitro techniques. The recent development of assays of more primitive progenitors in man (14) may provide the opportunity to further define the control mechanisms by which the neoplastic cells from the CML clone influence the expression of normal cells.

ACKNOWLEDGMENTS

This work was supported by grants CA16448, CA18029, CA20180, CA05826, GM 15253, and AM 19140 from the National Cancer Institute, the Institute of General Medical Sciences, and the Institute of Arthritis, Metabolism, and Digestive Diseases, the National Institutes of Health, and the Medical Research Service of the Veterans Administration.

REFERENCES

1. Fialkow, P. J., S. M. Gartler, and A. Yoshida. 1967. Clonal origin of chronic myelogenous leukemia in man. *Proc. Natl. Acad. Sci. U. S. A.* **58:** 1968–1971.
2. Fialkow, P. J., A. M. Denman, R. J. Jacobson, and M. N. Lowenthal. 1978. Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. *Am. J. Med.* **63:** 125–130.
3. Fialkow, P. J. 1980. Clonal and stem cell origin of blood cell neoplasms. *In* Contemporary Hematology/Oncology. J. Lobue, A. S. Gordon, R. Silber, and F. M. Muggia, editors. Plenum Publishing Corporation, New York. **1:** 1–46.
4. Singer, J. W., Z. A. Arlin, V. Najfeld, J. W. Adamson, S. J. Kempin, B. D. Clarkson, and P. J. Fialkow. 1980. Restoration of nonclonal hematopoiesis in chronic myelogenous leukemia following chemotherapy-induced loss of the Ph^1 chromosome. *Blood.* **56:** 356–360.
5. 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.
6. Singer, J. W., P. J. Fialkow, L. W. Dow, C. Ernst, and L. Steinmann. 1979. Unicellular or multicellular origin of

human granulocyte-macrophage colonies in vitro. *Blood.* **54:** 1395–1399.
7. 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.
8. Singer, J. W., J. W. Adamson, C. Ernst, N. Lin, L. Steinmann, S. Murphy, and P. J. Fialkow. 1980. Polycythemia vera: physical separation of normal and neoplastic committed granulocyte-macrophage progenitors. *J. Clin. Invest.* **66:** 730–735.
9. Fialkow, P. J. 1980. Studies with glucose-6-phosphate dehydrogenase on the origin of human neoplasms. *In* Viruses in Naturally Occurring Cancer. M. Essex, G. Todaro, and H. Zur-Hausen, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. **7:** 1171–1186.
10. Singer, J. W., P. J. Fialkow, L. Steinmann, V. Najfeld, S. J. Stein, and W. A. Robinson. 1979. Chronic myelocytic leukemia: failure to detect residual normal committed stem cells in vitro. *Blood.* **53:** 264–268.
11. Adamson, J. W., J. W. Singer, P. Catalano, S. Murphy, N. Lin, L. Steinmann, C. Ernst, and P. J. Fialkow. 1980. Polycythemia vera: further studies of hematopoietic regulation. *J. Clin. Invest.* **66:** 1363–1368.
12. Broxmeyer, H. E., A. Jacobsen, J. Kurland, N. Mendelsohn, and M. A. S. Moore. 1978. In vitro suppression of normal granulocytic stem cells by inhibitory activity derived from human leukemia cells. *J. Natl. Cancer Inst.* **60:** 497–503.
13. Olofsson, T. R., and I. Olsson. 1980. Suppression of normal granulopoiesis in vitro by a leukemia-associated inhibitor of acute and chronic leukemia. *Blood.* **55:** 975–982.
14. Fauser, A. A., and H. A. Messner. 1978. Granulo-erythropoietic colonies in human bone marrow, peripheral blood and cord blood. *Blood.* **52:** 1243–1247.