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Polycythemia vera. Physical separation of normal and neoplastic committed granulocyte-macrophage progenitors.

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Research Article

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Polycythemia Vera

PHYSICAL SEPARATION OF NORMAL AND NEOPLASTIC COMMITTED GRANULOCYTE-MACROPHAGE PROGENITORS

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ABSTRACT In previous studies of two patients with polycythemia vera (PV) who were heterozygous at the X-linked locus for glucose-6-phosphate dehydrogenase (G6PD), only A type enzyme was found in nonlymphoid blood cells. However, some erythroid and granulocytic colonies grown in vitro were type B and therefore arose from presumably normal progenitors. One patient had enough type B colonies (8%) that studies of the physical characteristics of normal and PV clonal colony-forming cells could be undertaken. When marrow cells were separated by velocity sedimentation at unit gravity, most PV clonal granulocyte-macrophage progenitors (CFU-C) (type A G6PD) sedimented between 6.4 and 7.2 mm/h, whereas most residual normal, type B CFU-C sedimented $\leq$5.9 mm/h ($P = 0.04$). When blood cells were separated over a discontinuous buoyant density gradient, PV clonal CFU-C equilibrated at densities < 1.065 g/ml, whereas residual normal CFU-C were found $\geq$1.065 g/ml ($P < 0.01$). PV clonal and residual normal erythroid burst-forming progenitors were not separable by either method. Thus PV clonal CFU-C are larger and less dense cells than are residual normal CFU-C.

INTRODUCTION

Previous studies have shown that polycythemia vera (PV)[1] is a clonal disorder originating in a stem cell common to at least erythrocytes, granulocytes, and platelets (1). These studies delineating cell lineage relationships and demonstrating the probable clonal origin of PV were made possible by use of the naturally occurring mosaicism at the glucose-6-phosphate dehydrogenase (G6PD) locus. Women heterozygous for the common gene ($\underline{Gd}^B$) and a variant such as $\underline{Gd}^A$ have two populations of cells; one synthesizing type B and the other type A enzyme. If a neoplasm originates in a single cell, all neoplastic tissue will have a single enzyme type. In a study of two G6PD heterozygotes with PV, normal tissues had both B and A enzymes in nearly equal amounts, but peripheral blood erythrocytes, granulocytes, and platelets had only a single enzyme, type A.

To determine whether normal committed stem cells persisted in these two patients, the G6PD isoenzyme types of individual erythroid and granulocytic colonies grown in semisolid media were determined (2). Although most of the colonies were type A, some colonies with B-type G6PD were found in cultures from both patients. This finding suggested that normal stem cells persisted in the disease and that their terminal maturation was suppressed in vivo. In this study of one of our patients, we have undertaken to determine whether normal and neoplastic committed stem cells differed in size or buoyant density.

METHODS

The clinical details of the patients course have been reported (1–3). Studies were done on three occasions starting 6 yr after diagnosis. The hematologic values at the times of study are given in Table I. Her treatment consisted of intermittent chlorambucil before the first two studies, however no drug was given for 2 mo before either study. At the time of the third study, the patient was receiving 1,000 mg/d of hydroxyurea. The patient was hospitalized at the Clinical Research

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[1] *Abbreviations used in this paper*: BFU-E, erythroid bursts; CFU-C, granulocytic colonies; D-PBS, Dulbecco's phosphate-buffered saline; G6PD, glucose-6-phosphate dehydrogenase; PV, polycythemia vera; Sv, sedimentation velocity.

TABLE I
Clinical Data

Study	Date	Hemoglobin	Leukocyte	Platelet	Treatment
		g/100 ml	*count/mm³*		
Unit gravity gradient	2/78	15.8	8,300	260,000	Intermittent chlorambucil (no medication for 2 mo before study)
Discontinuous density gradient	1/79	15.1	23,100	501,000	As above
Discontinuous density mixed gradient	11/79	16.0	10,500	720,000	Hydroxyurea, 1 g/d

Center of the University of Washington Hospital. After the patient's informed consent was obtained, marrow and peripheral blood samples were drawn into syringes containing preservative-free heparin and processed within 2 h.

Cell separation by unit gravity. PV marrow cells were enriched by preparation of a buffy coat and then were separated by velocity sedimentation at unit gravity using a modification of the method of Miller and Phillips (4, 5). The gradient was harvested by gravity in 18 fractions of 46 ml. An aliquot was removed from each fraction for cell counting and the remainder was centrifuged for 10 min (200 *g*, 4°C). The number of trypan blue dye-excluding nucleated cells was determined for each fraction and the cells then suspended in alpha medium (Flow Laboratories, Inc., Rockville, Md.). Fractions containing too few cells were pooled with an adjacent fraction and then plated for growth of progenitor cells giving rise to granulocytic colonies (CFU-C) or erythroid bursts (BFU-E).

Cell separation by discontinuous Percoll gradients. The blood was diluted twofold in Hanks' balanced salt solution and layered onto a 1.077 gm/ml Ficoll-diatrizoate solution (Teva Ltd., Jerusalem). The interface cells were harvested, washed twice in Hanks' balanced salt solution and then applied to a discontinuous density gradient made with solutions of colloidal silica coated with polyvinyl-pyrrolidone (Percoll; Pharmacia Fine Chemicals Inc., Piscataway, N. J.). In a "mixing" experiment, peripheral blood from a hematologically normal, Caucasian male volunteer was treated similarly. After being washed and counted, 25×10^6 interface cells from the PV patient and the normal subject were pooled before they were applied to the discontinuous density gradient.

Percoll, obtained as a sterile solution with a density of 1.130 g/ml, was adjusted to physiologic osmolarity by mixing nine parts Percoll and one part 10× Dulbecco's phosphate-buffered saline (D-PBS; Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.). The pH was adjusted to 7.2 with 0.1 N HCl. Further dilutions were made with 1× D-PBS to achieve the desired densities. The refractive index of each solution was tested before layering and the density was confirmed from a graph relating refractive index to measured density. Density was also checked on two occasions by direct measurement of each solution on an analytical balance using 0.5-ml class A volumetric pipettes. All measurements were within 0.002 g/ml of the calculated density. A 10-step discontinuous gradient was formed by carefully layering 1-ml aliquots of Percoll solutions of decreasing densities in a 16 × 125 mm plastic tube (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.). The density of the solutions decreased in 0.005 g/ml decrements from 1.090 g/ml at the bottom to 1.050 g/ml at the top. Approximately 5×10^8 cells were suspended in 1.0 ml of D-PBS 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. The fractions were washed once in 10 vol of D-PBS, resuspended in Eagles minimal essential medium, counted in a hemocytometer and then plated for colony growth.

Colony growth and analysis of G6PD type. Granulocyte colonies were grown in a plating mixture consisting of 20% fetal calf serum, 0.8% methylcellulose (Dow Corning Corp., Midland, Mich.) and a maximally stimulatory dose of phytohemagglutinin-conditioned medium (6). Erythroid colonies were grown under conditions previously described in the presence of 5 and 10 U/ml of sheep plasma erythropoietin (Step III, Connaught Laboratories, Willowdale, Ontario, Canada) (2). The plates were incubated 10–14 d in humidified 5% CO_2, 95% air incubators and the colonies were counted with an inverted microscope. Individual colonies were electrophoresed for G6PD as described (2). All colonies seen at ×25 in a culture dish or, if growth was excessive, in a quadrant of a dish, were electrophoresed.

RESULTS

Direct G6PD analysis of cell preparations. As in the prior studies (1, 2, 5), when erythrocytes, granulocytes, and platelets from the PV patient were tested directly only type A G6PD activity was found. As expected, the normal male had only type B activity.

Cell separation by unit gravity. Unit gravity velocity sedimentation separation of marrow cells was performed at a time when 8/101 CFU-C grown from unseparated marrow buffy coat cells were type B. Fig. 1 shows the cells per fraction from the PV patient and the mean values from three gradients performed on normal marrows. There was a shift in the modal sedimentation velocity (Sv) from 4.6 mm/h in the normal gradients to 7.4 mm/h in the PV gradient (Fig. 1). When granulocytic colonies were cultured from the fractions, the modal Sv for the CFU-C from the normal gradients was 5.4 mm/h, whereas that of the PV CFU-C was 7.2 mm/h (Fig. 1). When the G6PD isoenzyme types of individual colonies from gradient fractions were determined, the frequency of type B, presumably normal colonies, was 32% at Sv $\leq$ 5.9 mm/h, and fell to zero between 6.5 and 7.1 mm/h (Table II). 19% of the colonies at still higher Sv were type B.

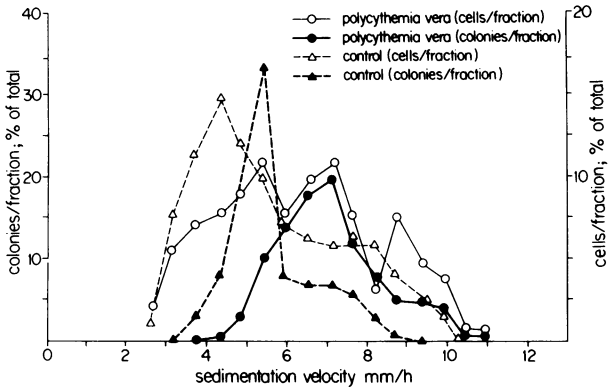

FIGURE 1 The mean nucleated cell and CFU-C profiles of three normal marrow samples and a marrow from the PV patient separated on unit gravity velocity sedimentation gradients. The values of this and all subsequent figures represent the percentage of the total harvest from each gradient.

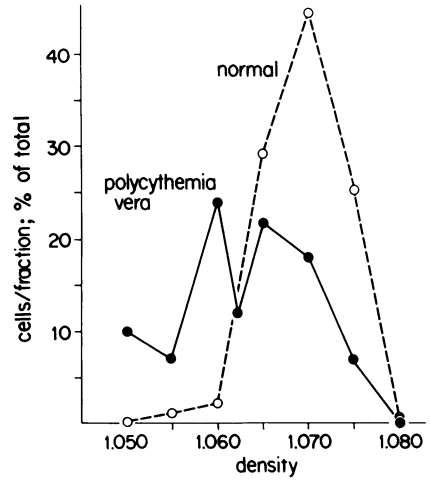

FIGURE 2 The mean cell profiles of mononuclear cell fractions from three normal individuals and the PV patient separated on discontinuous buoyant density Percoll gradients.

Since the patient's skin biopsy showed a 1:1 ratio of A to B enzyme, and previous studies have shown that the enzyme ratios of normal mesenchymal tissues such as skin and blood are the same (7), the frequency of normal colonies that type as A should equal that of B; therefore, the percent of normal colonies is twice the frequency of type B colonies. Thus, at the Sv of normal CFU-C of 5.4–5.9 mm/h, 64% of the colonies were normal. When the prevalence of type B colonies at Sv ≤ 5.9 and >5.9 mm/h were compared, the differences were statistically significant ($P = 0.04$; Fisher's exact test). When BFU-E were harvested for G6PD isoenzyme analysis from the fractions, the prevalence of type B colonies was approximately equal throughout and did not differ from the prevalence of type B colonies grown from unseparmarrow cells (Table II).

Cell separation by discontinuous Percoll gradients. Density gradient separation of peripheral blood cells was performed 6 mo later when the frequency of peripheral blood type B colonies had fallen to 1/46. The cell profile of the PV Percoll gradient and the mean cell profiles of three gradients from normal individuals are shown in Fig. 2. Most normal peripheral blood mononuclear cells equilibrate between 1.060 and 1.070 gm/ml with a modal value of 1.070 g/ml. Normal CFU-C equilibrated at densities between 1.060 and 1.070 g/ml with over 90% equilibrating ≥1.065 g/ml. In contrast, the profile of the Percoll gradient done with the PV cells showed a bimodal distribution with 56% of the cells equilibrating ≤ g/ml (Fig. 2). When CFU-C were grown from each gradient fraction and subsequently harvested for G6PD isoenzyme determinations, the data shown in Fig. 3 were obtained. The modal den-

TABLE II
Frequency of Type A and B G6PD in Colonies Grown from Marrow Cells Separated by Unit Gravity Velocity Sedimentation

Sv	G6PD type	
	A colonies	B colonies
CFU-C		
5.4–5.9	15	7*
6.5–7.1	11	0*
7.6–8.7	34	8
BFU-E		
5.4–5.9	36	3
6.5–7.1	21	3
7.6–8.7	21	1

* $P = 0.04$ by Fisher's exact test.

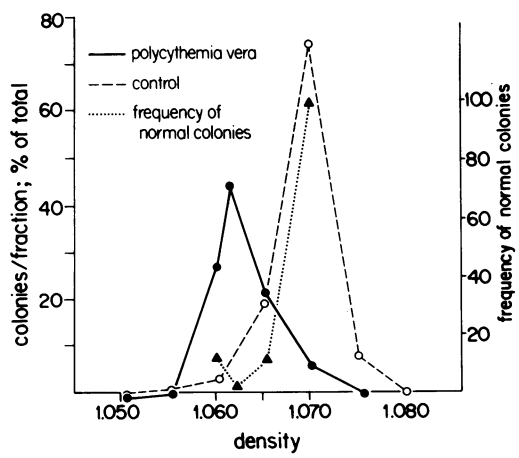

FIGURE 3 The mean peripheral blood CFU-C profiles from three normal individuals and the PV patient separated on Percoll gradients. The dotted lines represent the percentage of normal colonies at each density determined by G6PD.

TABLE III

G6PD Types of Colonies Grown from Blood Cells Separated on a Discontinuous Percoll Gradient

Density	G6PD type	
	A colonies	B colonies
g/ml		
CFU-C		
1.060	18	2
1.062	29	1*
1.065	22	3
1.070	4	4*
BFU-E		
1.060	13	0
1.070	11	0

* $P < 0.01$ by ψ^2 for the frequency of type B colonies observed at 1.070 g/ml compared to colonies at ≤1.065 gm/ml.

sity of PV CFU-C was 1.062 g/ml. At the modal density for normal CFU-C (1.070 g/ml), 50% of the colonies from the PV patient typed as B, indicating that almost none of the colonies were derived from the abnormal clone. At the modal density for PV CFU-C of 1.062 g/ml, only 1 of 29 colonies typed as B ($P < 0.01$) (Table III). When the isoenzyme types of BFU-E cultured from the same gradient were analyzed, no separation of normal BFU-E and those from the PV clone was observed (Table III).

Approximately 10 mo later no type B BFU-E or CFU-C were detectable in standard cultures of blood or marrow from the same patient. Therefore, an experiment was performed in which peripheral blood mononuclear cells from the type A PV patient and cells from a type B male were mixed and then separated on a Percoll gradient. Gradient separations were also performed on the PV cells and on the normal cells alone.

As shown in Table IV, the CFU-C concentrations in the PV and normal bloods were similar: $63 \pm 17/10^6$ and $50 \pm 15/10^6$ mononuclear cells, respectively. When the two mononuclear cell populations were mixed in a 1:1 proportion, 88 ± 16 colonies/10^6 cells were found. Since the colonies that grew in the plates with the unseparated mixed cells were not harvested for G6PD, no conclusion about the influence of the PV cells on normal colony growth can be made. The cell yields from the PV, the normal and the mixed gradients were 95, 50, and 63% of the cells applied. Ratios of the numbers of CFU-C actually recovered from the gradients to the maximal possible recoveries based on the CFU-C concentration in the unseparated samples were 4.01, 0.82, and 4.43 for the PV, normal and mixed gradients, respectively (Table IV). The cell profile from the mixed gradient appeared to be a simple summation of the PV and normal gradients (Fig. 4). As seen in Fig. 5, the modal density for the PV CFU-C was 1.060 g/ml, whereas for the concurrent normal it was 1.065 g/ml. The CFU-C profile of the mixed gradient also appeared to be a summation of the two single gradients when allowance is made for the striking increase in PV colonies found when cells were separated (Table IV).

Table V shows the G6PD isoenzyme analysis of individual colonies grown from the discontinuous gradient separation of equal mixtures of peripheral blood mononuclear cells from the PV patient and the normal male. At a density of 1.070 g/ml, 68% of the granulocytic colonies found were type B and thus originated from the normal cells. At the modal density for the PV cells of 1.060 g/ml, ~90% of the colonies arose from the PV clone ($P < 0.001$, χ^2). In contrast, no significant separation of normal and PV BFU-E was observed (Table V).

DISCUSSION

Previous studies of marrow regulation in G6PD heterozygotes with PV have shown that the disease appears to arise clonally in a progenitor common to the erythrocytes, platelets, and granulocytes (1). However, when marrow and peripheral blood cells were cultured under

TABLE IV

Cell Culture Results and Discontinuous Density Gradient Yields of Peripheral Blood Mononuclear Cells From the PV Patient, Normal Subject, and a 1:1 Mixture of Cells From Both

Cells	CFU-C concentration in unseparated blood mononuclear cells	Percent recovery of cells applied to gradient	Percentage of predicted CFU-C yield*
	colonies/10^6 cells plated (mean ± SD)		
PV	63±17	95	401
Normal	50±15	50	82
PV + normal (1:1 mixture)	88±16	63	443

* Percentage of predicted CFU-C yield was calculated as follows: total colonies cultured from the gradient divided by CFU-C/10^6 unseparated cells times number of cells applied to gradient times percentage of cells recovered divided by 100.

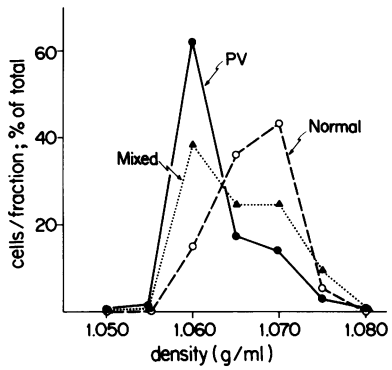

FIGURE 4 The mononuclear cell density profiles of a normal individual, the PV patient and an equal mixture of cells from the two separated on Percoll gradients.

appropriate conditions, normal committed stem cells were detected that gave rise to colonies that did not contain the G6PD type marking the neoplastic clone (2). The prevalence of normal BFU-E and CFU-C was high enough that had the colony-forming cells given rise in vivo to mature progeny, such cells should have been detectable in circulation. Thus, in some manner, the neoplastic clone appeared to suppress the maturation of normal stem cells.

Insight into the manner by which suppression of normal granulopoiesis might occur was obtained when marrow cells from two PV patients with a G6PD type A clone were incubated with high specific activity tritiated thymidine and then plated for CFU-C and BFU-E growth (3). The frequency of G6PD type B granulocytic colonies in both patients rose after exposure to tritiated thymidine. No change in the prevalence of normal BFU-E was seen. Statistical

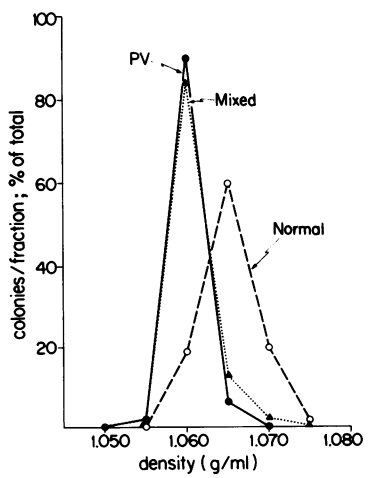

FIGURE 5 The profiles of CFU-C cultured from mononuclear blood cells from a normal individual, the PV patient and an equal mixture of cells from both separated on Percoll gradients.

TABLE V

G6PD Types of Colonies Grown From a Discontinuous Density Gradient Performed on a Blood Cell Mixture From the Type A PV Patient and a Type B Normal Subject

Density	G6PD type		
	A colonies	B colonies	AB colonies*
g/ml			
CFU-C			
1.060	25	2‡	2
1.070	8	17‡	0
BFU-E			
1.060	19	1§	4
1.070	7	4§	2

* For statistical purposes colonies that typed as AB were assumed to arise from 1 A and 1 B progenitor.
‡ $P < 0.01$; ψ^2.
§ Not significantly different.

analysis indicated that there was an absolute increase in normal CFU-C detected in cultures from patient 2. Thus, PV CFU-C cycled more rapidly than normal CFU-C and cycling cells from the neoplastic clone may have suppressed the proliferation of normal CFU-C in vitro. The increased expression of normal CFU-C but not BFU-E after the loss of cells synthesizing DNA suggests that early granulopoiesis and erythropoiesis are regulated differently and that suppression of normal stem cell expression occurs by different mechanisms.

In this study, we attempted to determine whether the physical properties of neoplastic and normal committed stem cells in PV are different as has been reported for CFU-C in chronic myelogenous leukemia (8). To examine this question, two different physical separation methods were employed; the first separating cells on the basis of their size and the second on the basis of their density.

When marrow cells from the PV patient were applied to the velocity sedimentation gradient, the modal Sv for PV and normal CFU-C differed significantly with values of 7.2 and 5.1 mm/h, respectively ($P < 0.01$). When the G6PD types of CFU-C from each fraction were tested, the majority of residual normal type B colonies were found to sediment ≤ 5.9 mm/h. This was identical to the previously determined modal Sv for normal CFU-C (5.9 mm/h). CFU-C sedimenting at 7.2 mm/h were all type A, although some type B, presumably normal CFU-C were found at higher SV, which possibly represented doublets rather than single cells. Thus the majority of residual normal CFU-C in this patient were smaller cells than CFU-C belonging to the dominant PV clone. When the overall frequency of nor-

mal CFU-C recovered from the gradient was calculated, it was found to be 19%, similar to the 16% predicted from results with unseparated marrow. This suggests that no factor(s) suppressing normal CFU-C growth was separated by this technique. In contrast to CFU-C, normal and PV clonal BFU-E did not separate on the gradient.

6 mo later, when only 1 of 32 peripheral blood CFU-C was G6PD type B, peripheral blood mononuclear cells were separated on a discontinuous density gradient using Percoll. CFU-C grown from Percoll fractions from the PV patient were shifted towards lighter densities with a modal value of 1.062 g/ml in the first experiment (Fig. 3) and 1.060 g/ml in the second (Fig. 5). Only 12 and 6% of CFU-C had densities ≥ 1.065 g/ml in the two gradients. In the first experiment, at 1.070 g/ml, equal numbers of A and B colonies were found; therefore 100% of the colonies were normal. At densities ≤ 1.065 g/ml, fewer than 10% of the colonies were normal. Thus, data from gradients indicated that the neoplastic CFU-C have abnormally light buoyant densities and, furthermore that the residual normal CFU-C had normal buoyant densities. This can be interpreted as further evidence of the "normalcy" of the residual type B stem cells. Normal and neoplastic BFU-E, unlike CFU-C, did not differ in buoyant densities.

Since normal CFU-C were not detected at the time of the final study, we mixed and then separated peripheral blood cells from a type B male and the PV patient. The gradient data obtained from mixing normal cells with those from the patient closely approximated those found earlier when both normal and PV committed stem cells were found in the patient's blood. Approximately 90% of normal CFU-C in the PV blood on 1/79 and type B CFU-C from the normal individual grown from the mixed gradient experiment equilibrated at densities ≥ 1.065 g/ml, whereas >90% of PV CFU-C equilibrated at densities < 1.065 g/ml on both occasions. However, unlike the results from the 1/79 gradient, which yielded 88% of the predicted number of granulocytic colonies, there was a 401% increase in the number of colonies harvested over the expected value. When PV cells were mixed with normal cells, applied to a gradient, and studied for G6PD after culture, the results suggest that no inhibition of normal CFU-C growth occurred. However, it is apparent that an interacting cell population(s) separated by the discontinuous density gradient was inhibiting proliferation of CFU-C from the PV clone in the unseparated samples. In view of the absence of inhibition of CFU-C growth from the normal subjects, these nonmutually exclusive hypotheses can be considered: (*a*) several populations of cells of different densities were required for inhibition, (*b*) the inhibition was restricted to autologous CFU-C, or (*c*) to CFU-C derived from the PV clone. Further studies are planned to clarify this issue.

The ability to separate PV clonal type A CFU-C from residual type B CFU-C on the basis of physical characteristics provides further evidence for the postulate that the type B CFU-C detectable earlier in the patient's course were in fact normal. Studies of G6PD heterozygotes with other diseases such as chronic myelogenous leukemia and acute nonlymphocytic leukemia need to be performed to determine if normal and neoplastic stem cells in these disorders are also physically separable.

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