DECAY-ACCELERATING FACTOR IS PRESENT ON PAROXYSMAL NOCTURNAL HEMOGLOBINURIA ERYTHROID PROGENITORS AND LOST DURING ERYTHROPOIESIS IN VITRO

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In the disease paroxysmal nocturnal hemoglobinuria (PNH),[1] erythrocytes and other blood elements have an acquired defect that renders them abnormally sensitive to the lytic action of complement. A number of erythrocyte membrane abnormalities have been shown to be associated with the PNH defect: (*a*) the PNH cell's membrane is a better acceptor of activated C3 than is the membrane of a normal cell (1); (*b*) the C3, once deposited on the membrane, is more stable than C3 deposited on a normal cell (2, 3); (*c*) the PNH cell membrane may allow more effective insertion of the late-acting lytic components than do normal cell membranes (4).

Recently, two intrinsic cell membrane proteins have been reported to influence the stability of complement enzymes and proteins deposited on normal human erythrocytes. CR1, the receptor for the complement components C4b and/or C3b, acts to control the degradation of these important protein fragments when deposited on cell surfaces (reviewed in 5 and 6). Recent work has suggested that erythrocytes (RBC) from patients with PNH have decreased red cell CR1 (7, 8); however, we have reported a patient with absent erythrocyte CR1 without evidence of a PNH-like red cell defect (8). A second protein, DAF, originally described as a factor accelerating the decay of the classical pathway C3 convertase, recently has been found to accelerate the destruction of the alternative pathway convertase as well (8). Work from two laboratories has suggested that this protein is partially or completely absent from the membranes of PNH RBC (8, 9). A total of three patients has been reported with absent DAF in their PNH RBC population. In addition, antibody to this protein has been reported to induce a PNH-like abnormality of normal cells (8). Defective regulation of the membrane-bound convertase would at least partly explain the abnormal sensitivity of PNH RBC to complement-mediated lysis.

[1] *Abbreviations used in this paper:* BFU-E, erythroid burst-forming cell; CFU-E, late erythroid colony-forming cell; DAF, decay-accelerating factor; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; PNH, paroxysmal nocturnal hemoglobinuria; RBC, red blood cell.

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TABLE I
Clinical Status of PNH Patients

Patient	Age	Sex	Status	Percent BM cellularity	Percent PNH RBC	Percent DAF^- RBC
3	33	F	Moderate pancytopenia	80	33	43
4	40	M	Mild thrombocytopenia; recurrent hemolytic crises	40	17	40
10	34	M	Mild hemolysis; androgen Rx	75	28	29
14	30	F	Moderate pancytopenia	70	22	10
15	26	M	Moderate pancytopenia; recurrent hemolytic crises; steroid Rx	70	37	44
17	26	M	Recovered aplastic anemia; mild pancytopenia	10	19	21

Patient numbers refer to a larger series of individuals with PNH to be reported.[2] All patients had positive Ham tests. PNH RBC were determined spectrophotometrically. DAF^- RBC were determined using a specific anti-DAF antiserum and flow microfluorometry.

We used a specific antiserum to DAF to study the generation of cells lacking DAF during erythropoiesis. DAF expression was measured on the erythroid progenitors, their in vitro progeny normoblasts, and the circulating RBC of the PNH patients.

Materials and Methods

Patients and Controls. The clinical and laboratory features of the patients in the current study are described elsewhere[2] and the corresponding patient identification numbers are used here. All six patients (Table I) had a positive Ham test, and variable proportions of PNH cells could be measured in the circulation by a quantitative, spectrophotometric analysis of complement lysis (10). All patients showed evidence of either chronic or recurrent episodes of hemolysis or bone marrow failure manifested by pancytopenia. At the time of study one patient was receiving corticosteroids, and another, anabolic steroids; four patients were untreated. Studies were reproduced in part on separate occasions in two of the cases studied. Individuals who served as controls were normal volunteers. Protocols for obtaining blood and bone marrow from patients and volunteers were approved by the National Heart, Lung, and Blood Institute institutional review board.

Tissues. Peripheral blood was collected in preservative-free heparin (Forest Pharmaceuticals, Inc., St. Louis, MO), 1,500 U/30 ml. Bone marrow was aspirated from the posterior iliac crest directly into syringes containing 1.5 ml Iscove's modification of Dulbecco's medium with 100 U/ml of heparin or, in some cases, into 1.5 ml of EDTA in phosphate-buffered saline (PBS) to a final concentration of 10 mM. Mononuclear cells were isolated by density gradient sedimentation on Ficoll (Lymphocyte Separation Medium; Litton Bionetics, Inc., Charleston, SC).

Erythroid Colony Cultures. Bone marrow mononuclear cells were cultured to detect the primitive erythroid progenitor, the erythroid burst-forming cell (BFU-E), and the more mature progenitor, the erythroid colony-forming cell (CFU-E), in methylcellulose as described.[2] CFU-E-derived colonies were recognized by their characteristic morphology at day 8; BFU-E-derived bursts consisted of a minimum of three clusters at day 18. Erythroid colonies were removed from culture using a 10 μl vol micropipette or a tapered Pasteur pipette.

[2] Moore, J. G., R. K. Humphries, M. M. Frank, and N. S. Young. Manuscript submitted for publication.

Flow Microfluorometry. Flow microfluorometric analysis was performed on three different cell populations: peripheral blood erythrocytes, bone marrow mononuclear cells, and normoblast progeny in colonies removed from methylcellulose cultures. The characteristics of rabbit anti-DAF antibody used in these studies have been reported elsewhere (8). The antiserum recognizes a 73,000 mol wt structure on all normal RBC that represents the DAF molecule. Binding of this antiserum renders normal red blood cells PNH-like, making them susceptible to lysis by acidified serum. The antiserum was first absorbed with PNH RBC membranes from patient 3 to remove the minor contaminating antibody specificity directed against the complement receptor CR1. The fluorescein-conjugated mouse anti-rabbit IgG (HyClone Laboratories, Logan, UT) was repeatedly absorbed with normal peripheral blood red cells to reduce nonspecific binding. RBC (2×10^7 to 6×10^7), bone marrow mononuclear cells (4×10^6), or individual bursts (150–60,000+ cells per colony) were incubated with anti-DAF diluted 1:10 in PBS for 30 min at 4°C, washed twice in PBS, and incubated with anti-rabbit IgG at optimal concentrations, followed by two final washes and suspension in PBS. Erythrocytes were analyzed and bone marrow mononuclear cells were analyzed and sorted using a fluorescence-activated cell sorter (FACS II; Becton Dickinson Immunocytometry Systems, Mountain View, CA). For technical reasons, the small numbers of cells that constituted mature erythroid colonies were analyzed using an Epics V (Coulter Electronics, Hialeah, FL).

Erythroid progenitor cells have been reported to lack Fc receptors (11). In confirmation of these findings, we found that an undetectable number of bone marrow mononuclear cells bound aggregated human IgG and fluoresceinated anti-human IgG (data not shown). To control for nonspecific binding of rabbit antibodies, human bone marrow mononuclear cells from patient 14 were incubated with an irrelevant rabbit antiserum and fluoresceinated anti-rabbit IgG, sorted by flow cytometry, and the antibody-binding and -nonbinding fractions cultured for the presence of erythroid progenitors. No hematopoietic colony-forming cells were found in the positive fraction of cells (data not shown). In other experiments, the same cell population incubated with fluoresceinated antibody alone served as a control.

Results

Detection of DAF$^-$ RBC in PNH Blood. To determine cell surface DAF expression, RBC were incubated with a specific rabbit antiserum to DAF followed by a fluoresceinated anti-rabbit IgG antibody and analyzed using a FACS. Two discrete populations of RBC were detected in all six patients with PNH (Fig. 1, *A* and *C*). The less fluorescent population of DAF$^-$ cells coincided with the negative control consisting of RBC exposed to the second antibody only (Fig. 1 *F*). In contrast, a single, DAF$^+$ population of RBC was observed in normal blood (Fig. 1 *E*).

Relationship of Absence of DAF to Complement Sensitivity. The amount of immunoprecipitated DAF has been correlated with the relative sensitivity of different populations of PNH RBC (9). To test the relationship between the absence of DAF and the sensitivity of RBC to lysis in acidified serum, we measured the size of the DAF$^+$ and DAF$^-$ RBC populations before and after complement lysis (Figs. 1 and 2). In all patients tested, incubation in acidified serum under optimal conditions for lysis of PNH cells resulted in reduction in the proportion of DAF$^-$ cells; the average reduction in relative DAF$^-$ populations was 52 ± 5%, as calculated from the respective ratios of negative cells. In no case was the DAF$^-$ population completely eliminated by prior acidified serum treatment.

DAF Expression of Erythroid Progenitor Cells. A variable proportion of PNH bone marrow cells was DAF$^-$ (25–79%, $x \pm$ SEM = 40 ± 11%; normal range, 5–20%, $x \pm$ SEM = 9 ± 5%). As hematopoietic stem cells are extremely infrequent

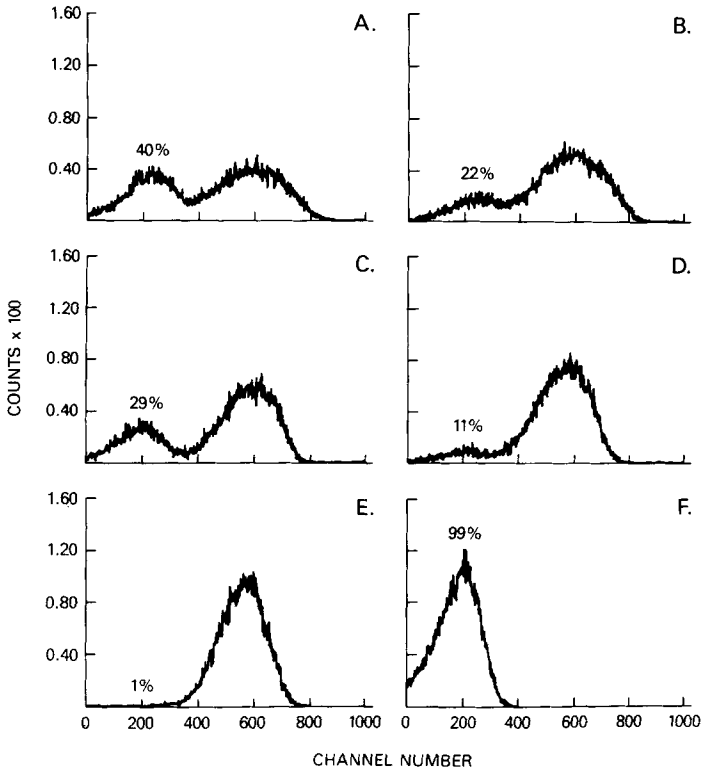

FIGURE 1. DAF expression on erythrocytes. Erythroyctes were incubated with a specific rabbit antiserum to DAF followed by a fluoresceinated anti-rabbit IgG antibody, and then analyzed by flow microfluorometry (see Materials and Methods). Two discrete populations of red cells were detected in all six PNH patients; data from two representative patients are shown in *A* and *C*. The population of DAF^- cells coincided with the negative controls consisting of red cells exposed only to the second antibody (*E*). Incubation of the patients' red cells in acidified serum before analysis reduced the DAF^- population (*B*, *D*). A single DAF^+ population of RBC was observed in normal blood (*F*).

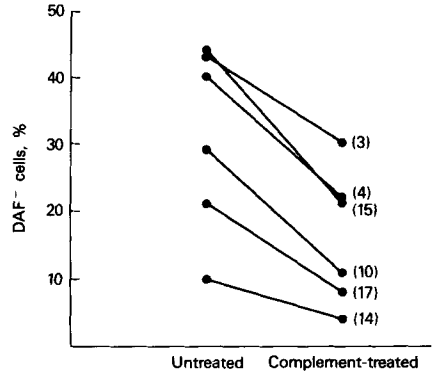

FIGURE 2. Relationship of the absence of DAF to complement sensitivity. To test the relationship between the absence of DAF and the sensitivity of red cells to lysis in acidified serum, the DAF^+ and DAF^- RBC populations were measured before and after complement-mediated lysis. Data from six patients studied have been plotted schematically; patient numbers are shown in parentheses. Incubation in heat-treated serum did not alter the proportion of DAF^- cells (data not shown).

in the bone marrow, we tested for the presence of DAF on erythroid progenitors using the FACS. PNH and normal bone marrow mononuclear cells labeled with anti-DAF were separated by flow microfluorometry, and the DAF^+ and DAF^- populations each cultured in methylcellulose for the detection of erythroid colonies. All erythroid colonies were grown from the DAF^+ populations of both normal and PNH bone marrow mononuclear cells, indicating that the BFU-E and CFU-E progenitor cells expresed the DAF antigen (Table II).

DAF Expression on Normoblasts that Develop In Vitro. Failure to detect a discrete population of DAF^- erythroid progenitors in PNH bone marrow suggested that DAF might be lost during erythroid differentiation. To test for this possibility, individual bursts, representing the progeny of a single erythroid progenitor cell, were removed from culture and analyzed for DAF expression (Fig. 3, Table III). The mature erythroid progeny of bursts grown from normal bone marrow were a single population of DAF^+ cells (Fig. 3*E*). In contrast, normoblasts from PNH bursts showed two discrete populations of cells by the criterion of DAF positivity, and most cells were DAF^-. Of the total progeny cells in three PNH patients analyzed, 79 ± 4% were DAF^-. Only 3 of 27 bursts analyzed, one in each patient, showed >50% DAF^+ or normal cells. The occurrence of DAF^- progeny in colonies derived from DAF^+ progenitors indicated

TABLE II
DAF Expression on Erythroid Progenitors

Patients	Progenitor	Colonies per 10^5 mononuclear cells	
		DAF^+	DAF^-
10	CFU-E	41	0
	BFU-E	3	0
14	CFU-E	17	0
	BFU-E	7	0
15	CFU-E	29	0
	BFU-E	23	0
17	CFU-E	89	0
	BFU-E	20	0
Normals			
1	CFU-E	146	0
	BFU-E	116	0
2	CFU-E	92	0
	BFU-E	87	0

Bone marrow mononuclear cells were separated by a FACS after labeling with anti-DAF antiserum and fluorescein isothiocyanate-conjugated anti-rabbit IgG. The DAF^+ and DAF^- cells were plated in methylcellulose to determine the proportion of erythroid progenitors (BFU-E and CFU-E) in each population. To control for loss during sorting experiments, colonies obtained were compared to cultures of unsorted cells for each of the persons tested and there was no significant difference (recovery was 94 ± 11%).

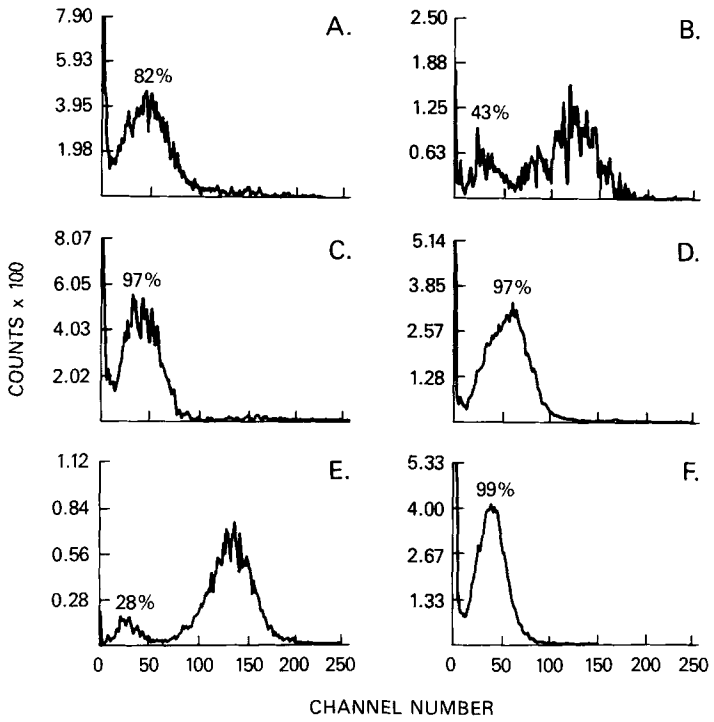

FIGURE 3. DAF expression or normoblasts that develop in vitro. Individual bursts, containing mature normoblasts, were removed from methylcellulose culture of bone marrow mononuclear cells on day 18 using a 10-μl micropipette and incubated with a specific antiserum to DAF, followed by a fluoresceinated anti-rabbit IgG, and then analyzed for DAF expression by flow microfluorometry. A representative burst from a normal person contains mostly DAF^+ cells (*E*). The small number of apparently DAF^- in the normal burst are probably artifactual, resulting from the inability to completely remove methylcellulose by washing of this small number of cells. In contrast, the normoblasts within individual bursts in PNH patients were mostly DAF^- (*A*, *B*, *C*, *D*), corresponding to cells exposed to second antibody alone (*F*).

that DAF expression had been lost, and this PNH characteristic acquired, during in vitro erythropoiesis.

The RBC of patients with the rare hematologic disorder termed HEMPAS (hereditary erythroblastic multinuclearity with positive acidified serum test) resemble PNH RBC in their sensitivity to antibody-mediated lysis by complement. However, in contrast to DAF expression on cells from patients with PNH, DAF expression on HEMPAS RBC, bone marrow mononuclear cells, and progeny normoblasts in vitro was normal (data not shown).

Discussion

In these studies we have used flow microfluorometry in combination with a highly specific polyclonal anti-DAF antibody to precisely quantitate the expression of DAF on circulating RBC, bone marrow mononuclear cells, erythroid progenitors, and their in vitro progeny. Blood samples from normal individuals showed only DAF^+ (or normal) RBC. The DAF^- RBC were partially eliminated by prior incubation with acidified serum, confirming the abnormal sensitivity of

TABLE III

DAF Expression on Normoblasts from Individual Bursts

	Percent PNH DAF^+ (No. of cells)		Percent normal DAF^+ (No. of cells)
Patient 4	5 (1,829)	Normal 1	71 (3,170)
	9 (1,428)		80 (1,339)
	12 (2,242)		84 (7,806)
	20 (595)		86 (23,028)
	21 (542)		89 (57,656)
	21 (3,120)		96 (805)
	24 (489)		
	26 (2,448)		
	33 (2,487)		
	83 (10,000+)		
$x \pm$ SEM	25 ± 7%		84 ± 3%
n	10		6
Patient 14	9	Normal 2	73
	11		81
	24		89
	31		
	33		
	33		
	64		
$x \pm$ SEM	19 ± 7%		81 ± 8%
n	7		2
Patient 17	3 (3,026)	Normal 3	68 (714)
	3 (19,274)		72 (2,545)
	4 (1,027)		73 (4,203)
	7 (2,964)		82 (4,203)
	8 (1,158)		
	10 (289)		
	14 (1,161)		
	15 (256)		
	18 (188)		
	57 (939)		
$x \pm$ SEM	14 ± 5%		73 ± 3%
n	10		4

Mature erythroid bursts were removed from methylcellulose culture and subjected to flow microfluorometry after staining with anti-DAF antiserum and anti-rabbit IgG fluorescein isothiocyanate. Results in three patients and three concurrent normal controls are expressed as the proportion of DAF^+ cells in each burst tested; shown in parentheses are the number of normal blasts in each burst.

DAF^- cells to acid hemolysis. The resistance of some DAF^- RBC to complement lysis might be due to suboptimal conditions for complement-mediated lysis or may reflect the presence of other fluid phase or RBC membrane-bound regulatory mechanisms.

DAF was also found expressed on all of the bone marrow mononuclear cells of normal individuals. In the PNH patients, both DAF^+ and DAF^- populations of mononuclear cells were found in the bone marrow (DAF^- PNH marrow cells, $x \pm SEM = 40 \pm 11\%; 9 \pm 5\%$). Surprisingly, all of the erythroid progenitor cells from both normals and PNH patients, as detected by clonogenic and highly specific colony assays, were present in the DAF^+ population, and therefore the erythroid progenitor cell in PNH was phenotypically normal by the criterion of DAF expression. The normoblast progeny obtained by in vitro culture of erythroid progenitor cells from normal individuals were mostly DAF^+ by flow microfluorometric analysis. However, unlike the in vitro findings with normal individuals, the phenotypically normal progenitors from PNH patients gave rise to mostly DAF^- or PNH-type normoblasts. Therefore, loss of DAF and acquisition of this PNH-associated defect were shown to be developmental phenomena. A residual normal population of erythroid progenitors may have been indicated by the single near-normal burst, containing mostly DAF^+ normoblasts, in each patient.

Our results using DAF as a marker of the PNH phenotype during in vitro erythropoiesis confirm our earlier experiments using acidified serum lysis.[2] In those studies, unlike Dessypris et al. (12), we were unable to detect a large proportion of hematopoietic progenitors in PNH bone marrow susceptible to acidified serum or isotonic sucrose lysis. Analogous to the results with DAF expression, experiments using complement-mediated lysis showed that erythroid progenitors that do not lyse in acidified serum give rise in vitro to normoblast progeny that are abnormally susceptible to complement lysis. Therefore, by both the DAF phenotype and the functional complement sensitivity defect, phenotypically normal progenitor cells from PNH bone marrow generate PNH-type progeny cells in vitro.

The loss of DAF and acquisition of complement sensitivity during erythropoiesis by PNH progenitor cells is analogous to a few previously described alterations in cell function and membrane phenotype detectable in vitro. For example, synthesis of fetal hemoglobin as the major globin species is a capacity of cells derived from the more primitive erythroid progenitor, the BFU-E, and not of normoblasts grown from the later progenitor, the CFU-E (13). Similarly, the HLA-DR antigen is expressed on BFU-E but is not present on CFU-E or more mature erythroid cells (14). However, the loss during differentiation of a cell surface marker and related functional capacity in association with a specific disease is, thus far, unique to PNH.

Only one burst of ~10 analyzed in each PNH patient contained mainly normal or DAF^+ progeny. In the patients' blood, however, the majority of RBC were normal, with PNH cells representing 17–37% of the total cells. Thus, the proportion of PNH RBC detected in the circulation of patients was always much lower than the proportion in the bone marrow, presumably reflecting the selective destruction in the bone marrow of both mature and immature erythroid cells, perhaps by a complement-mediated mechanism. These results are consistent with a model in which all the erythroid progenitors in PNH are phenotypically normal by the criteria of DAF positivity and resistance to complement, but only a minority (~10%) are genotypically normal, as reflected by their ability to give

rise to entirely normal progeny. The genotypically abnormal PNH cells produce the majority of progeny in tissue culture, either because they have a growth advantage or, more likely, because their progeny are not lysed as they are in the bone marrow of a patient. The patient's erythron, therefore, may be dependent for maintenance on a normal stem cell pool of very limited size. In another report,[2] we measured very low numbers of progenitor cells and also a remarkably high cell cycling rate of PNH bone marrow BFU-E, even in comparison with other hemolytic diseases. The demands placed on normal progenitor cells indicated by this rapid turnover and small pool size may provide the laboratory explanation for the close clinical relationship between PNH and aplastic anemia.

Based on studies of a limited number of PNH patients heterozygous at the G6PD locus (15, 16), the generation of the PNH population of RBC has been characterized as clonal. Our data clearly do not contradict such a somatic cell genetic defect. In other clonal erythropoietic disorders, the presence of functionally distinct red cell populations also has not correlated with similarly distinct populations of progenitor cells. For example, individual primitive erythroid stem cells in polycythemia vera generate both erythropoietin-independent as well as normally erythropoietin-dependent progeny (17). Studies of DAF synthesis in developing erythroid cells should permit a more precise localization of the defect in PNH cells and perhaps allow modulation of DAF expression both in vitro and in vivo.

Summary

A glycoprotein that regulates the deposition of C3b on the erythrocyte surface, called decay-accelerating factor or DAF, is absent from the red blood cells (RBC) of patients with paroxysmal nocturnal hemoglobinuria (PNH), explaining in part their abnormal sensitivity to complement. We used a specific antiserum to DAF, flow microfluorometry, and clonogenic assays for erythroid progenitor cells to study PNH erythropoiesis in vitro. By fluorescence-activated cell sorter analysis, all RBC from normal individuals are DAF^+. In contrast, the RBC of six patients with PNH showed discrete populations of DAF^- cells (10–44%; $x \pm$ SEM = 31 $\pm$ 6%). The DAF^- RBC population was partly eliminated by prior acidified serum lysis. To determine whether erythropoietic progenitors expressed DAF, bone marrow cells were sorted by flow microfluorometry and the separated DAF^+ and DAF^- populations then cultured in vitro. In two normal individuals, but also in six patients with PNH, erythroid colonies formed only from cells in the DAF^+ fraction. However, a variable proportion of the normoblast progeny of these DAF^+ progenitor cells from patients with PNH was DAF^-. Individual bursts removed from cultures of PNH bone marrow showed two discrete populations by fluorescence; the majority of normoblasts were DAF^-, only 3 of 27 individual bursts had >50% DAF^+ cells, and in three patients, DAF^- normoblasts averaged 79%. In contrast, the progeny of individual bursts from normal individuals comprised a unimodal DAF^+ population. In each PNH patient, one normal burst (>80% DAF^+ normoblasts) was detected, possibly reflecting a normal residual population of erythroid progenitors. By the criterion of DAF expression, there was no evidence of separate populations of normal and PNH

type progenitor cells. The phenotypically normal erythroid progenitors of PNH bone marrow acquire the PNH characteristics during differentiation in vitro.

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