Complement Sensitivity of Paroxysmal Nocturnal Hemoglobinuria Bone Marrow Cells

By Jon Turnen, Lanning B. Kline, Joseph W. Fay, Daniel C. Scullin, Emily G. Reisner, Wendell F. Rosse, and Andrew T. Huang

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired disorder in which erythrocytes, granulocytes, and platelets are defective, as shown by increased susceptibility of RBCs, WBCs, and platelets to complement-mediated lysis in vitro. The purpose of this study is to determine the sensitivity to complement lysis of PNH and non-PNH erythroid and myeloid precursors using the release of $^{51}$Fe and myeloperoxidase as specific markers to monitor the lytic action of complement on erythroid and myeloid cell precursors, respectively. Erythroid cell precursors in four of four PNH patients demonstrated increased sensitivity to complement-mediated lysis. Myeloid cell precursors in four of five PNH patients also exhibited increased sensitivity to complement and antibody. In addition, CFU-c growth was below normal in the marrow of seven PNH patients. These findings support the hypothesis that the defect in PNH occurs at the level of the hematopoietic stem cell.

PNH (paroxysmal nocturnal hemoglobinuria) was originally described by Strubing as a hemolytic anemia, characterized clinically by the occurrence of nocturnal hemoglobinuria.1,2 The usual clinical presentation is chronic hemolysis fluctuating in severity and often accompanied by hemoglobinuria. Viral infections, sepsis, transfusions, surgery, or strenuous exercise may cause acute exacerbation of the disease with brisk hemolysis. Patients with PNH are also particularly prone to venous thromboses.3 Presently, PNH is thought to be a more complex disorder that may involve abnormal hematopoiesis with defective or deficient production of all three cell elements: RBC, WBC and platelets.

The major cellular defect in PNH is believed to be a membrane abnormality that confers upon the cell an increased sensitivity to the lytic action of complement.4 In this disease, there exists a population or populations of red cells more susceptible to complement-mediated lysis than coexisting normal cells.5-8 To account for the increased complement sensitivity, it has been demonstrated that PNH red cells bind more C3 than normal cells, and that this by the terminal components of complement, C5–9, is more efficient.9

The earliest erythroid cells thus far found to have this abnormality are the reticulocytes in circulating blood.6,14

A similar membrane abnormality appears to be present in granulocytes. In most patients, a complement-sensitive population may be demonstrated in vitro.15 PNH granulocytes exhibit impaired phagocytosis and chemotaxis in the presence of activated complement.16,17 Cytochemical staining for leukocyte alkaline phosphatase (LAP) shows that the mean LAP of granulocytes in patients with PNH is significantly below normal.17,18 Craddock et al. have reported that PNH granulocytes, after exposure to activated complement, migrate poorly toward chemotaxins; but those containing normal quantities of LAP are capable of normal chemotaxis.17

In addition, PNH platelets express a membrane abnormality similar to that present in red cells and granulocytes. Increased complement-mediated lysis of PNH platelets has been demonstrated in vitro.19 Subsequent to the activation of complement by either the classic or alternative pathways, serotonin is released more readily in PNH than in normal platelets. Since most major thrombotic phenomena occur during periods of complement activation; the increased complement sensitivity of the PNH platelet (membrane) may account for the incidence of thrombosis in this disease.20,21

The trilineal demonstration of increased sensitivity to the lytic action of complement, in addition to the association seen in PNH with suspected stem cell disorders, including aplastic anemia,22,23 myelogenous leukemia,24-26 myelosclerosis,27,28 and erythroleukemia,29 suggests a clonal disorder of the marrow, in which a somatic mutation may occur at the level of the hematopoietic stem cell.30 However, there has been no direct evidence demonstrating such abnormality of marrow precursor cells, an essential step in the testing of this hypothesis.

The purpose of this investigation is to determine the complement sensitivity of PNH and non-PNH erythroid and myeloid precursors using the release of $^{51}$Fe and myeloperoxidase, respectively, as specific markers to monitor the lytic action of complement.

From the Division of Hematology-Oncology, Department of Medicine, Duke University Medical Center, Durham, N.C.

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Address reprint requests to Andrew T. Huang, M.D., Division of Hematology-Oncology, Department of Medicine, Duke University Medical Center, Durham, N.C. 27710.

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We have also performed PNH marrow culture studies to further investigate the in vitro growth characteristics in this disease.

**MATERIALS AND METHODS**

**Patients**

The bone marrow of 13 patients with the diagnosis of PNH and of 14 individuals with normal bone marrow was studied. Summaries of the PNH patients' clinical histories, red cell and laboratory studies are outlined in Table 1.

**Erythroid Cell Precursor Studies**

**Preparation of Cells**

After informed consent was obtained, sterile bone marrow specimens (3-25 ml) were obtained by aspiration from the sternum or posterior iliac crest into syringes primed with preservative-free heparin. The marrow specimens were passed through a 22-gauge needle to remove or break up particles, and placed in 15 ml of sterile alpha-medium (GIBCO, Grand Island, N.Y.) in conical tubes.Thirty percent (v/v) of Plasmagel (Lab. Roger Bellon, Neuilly, France) was added, and the marrow sample was allowed to stand at room temperature for 1 hr to separate RBCs from marrow cells. The supernatant radioactive culture medium was discarded, allowed to stand at room temperature for 1 hr. Each incubation was centrifuged at 200 g for 10 mm. From the centrifuged material, 5-10 ml of supernatant fluid was kept as a source of transferrin and the rest was discarded. The cell button from the centrifuged material was suspended in 50 ml of McCoy's 5a modified medium (GIBCO, Grand Island, N.Y.) with 15% fetal calf serum and 250-mI Bellco flask. To the source of transferrin, 10 % of 59Fe-ferrous citrate (13 mCi/g Fe; New England Nuclear, Boston, Mass.) was added, allowed to stand at room temperature for 1 hr, and then added to the marrow culture. The specific activity of 59Fe added to the cell culture was 0.33 mCi/ml. The total nucleated cell count after plasmagel treatment and prior to incubation was in the range of 1.5-4.5 × 106 cells in 50 ml of media. After overnight incubation in a CO2 incubator, the marrow culture was removed from the flask, and the supernatant radioactive culture medium was discarded.

**Purification of Erythroid Precursors**

The cells were first subjected to hypotonic lysis twice to destroy reticulocytes and mature red cells. The remaining cells were then washed twice in 0.9% buffered saline (pH 7.4) and once in Veronal-buffered saline (VBS). Prior to the final wash, aliquots of cells were pipetted into individual test tubes. Following centrifugation the final cell suspension volume was adjusted to 0.4 ml. The cell count of nucleated erythroid precursors per test tube was in the range of 3.5-8.5 × 106 cells.

**Antibody**

Human anti-l antibody [anti-l (Step.)] with high hemolytic potency was obtained from a patient with cold agglutinin disease and kindly provided by Dr. and Mrs. J.H. Crookston of Toronto, Ontario, Canada. This serum was stored at −20°C and thawed just prior to use. Fresh human serum (type AB, Rh positive) was utilized as the source of complement and was frozen at −90°C. Just prior to the complement lysis sensitivity test (CLS), the serum was thawed and the required dilutions were prepared in VBS.

**Assay**

Equal volumes (0.4 ml) of cells, antibody, and complement (in serial dilutions) were mixed together in individual test tubes and incubated at 0°C for 15 min, followed by incubation at 37°C for 1 hr. Each incubation was centrifuged at 200 g for 10 min and the cell button and supernatant fluid separated.22 Radioactivity in the cell button and that released into the supernatant fluid were counted in a Packard gamma counter and the percent lysis calculated according to the formula:

$$\text{Percent lysis} = \frac{\text{cpm in supernatant fluid}}{\text{cpm in cells before CLS or cpm in cell button} + \text{cpm in supernatant fluid}} \times 100\%$$

The percent lysis obtained from cells that were not treated with a combination of antibody and complement was considered as background and was subtracted from that calculated in the experimental
sample. Cell lysis from treatment with antibody alone or complement alone was not different from the background.

**Autoradiography**

Autoradiographs were prepared by spreading marrow cells on glass slides, which were then air-dried and fixed in absolute methanol for 10 min. Slides were exposed to Ilford L-4 emulsion (Essex, England) for 40 days. Slides were developed in Microdot-X (Eastman Kodak Co., Rochester, N.Y.) 1:1 at 20°C for 5 min, dipped in acetic acid stop bath, and fixed 5 min with 20% sodium thiosulfate at 20°C. Slides were then rinsed thoroughly in distilled water and air-dried. Wright's stain was used subsequently to facilitate study of the cell morphology.

**Myeloid Cell Precursor Studies**

**Cell Preparation**

Bone marrow specimens were diluted in 15 ml of alpha-media in sterile conical tubes. Centrifugation at 1500 g for 10 min was followed by aspiration of the buffy element, resuspension, and washing with alpha-media. Cells (10⁹) were layered atop a step-wise bovine serum albumin (BSA) gradient (17% and 25.2%) and centrifuged at 3800 g for 45 min to enrich for myeloid cell precursors and to eliminate contaminating peripheral granulocytes. Cells at the interface above 25.2% BSA (differential count, see Results) were removed, washed twice with alpha-media, and divided into 7 aliquots (1-6 x 10⁶ cells in 200 μl/tube).

**Antibody**

Because of insufficient supplies of high potency anti-I antisera at the time the myeloid cell study was being done, a polyclonal anti-HLA antibody obtained from an alloimmunized patient was used. This antibody, like other anti-HLA antibodies, required presence of complement for cytolysis. This serum, ineffective only in patients with A3, B7 HLA type, was used in the initial experiments. Antilymphocyte globulin, capable of lysing marrow cells and lymphocytes, was used in subsequent experiments when a patient with A3, B7 HLA type was being studied (prepared from horses and kindly donated by Dr. H. Seigler of Department of Surgery, Duke University Medical Center).

**Complement**

Commercial baby rabbit serum was utilized as a source of complement in these experiments as recommended by the HLA Typing Laboratory of our institution (Pel-Freeze, Rogers, Ark.). The serum was stored at −110°C and thawed just prior to use.

**Procedure**

Antibody (200 μl) was added to each tube containing 1-6 x 10⁶ cells in 200 μl for 20 min at room temperature. Each tube was centrifuged at 200 g for 10 min and the supernatant fluid removed. Complement (500 μl), serially diluted in alpha-medium, was added to the tubes, which were then vortexed and incubated for 30 min at 37°C. The tubes were again centrifuged at 200 g for 10 min and then placed on ice.

**Spectrophotometric Myeloperoxidase Assay**

Myeloperoxidase (MPO, an enzyme present in large amounts in myeloid cells from myeloblasts to mature neutrophils) is a hemoprotein catalyzing the oxidation by hydrogen peroxide of a number of substrates, such as ascorbate, ferrocyanide, cytochrome C, and the leuco form of many dyes. The assay used in this study utilizes 4-aminonitrophenyl hydrogen peroxide (0.0017 M) was prepared by diluting 1 ml of 30% H₂O₂ (Merck Superoxol) to 100 ml with glass-distilled water. One milliliter of this solution was further diluted to 50 ml with 0.2 M potassium phosphate buffer, pH 7.0. The hydrogen donor solution was prepared by dissolving 810 mg phenol in 40 ml glass-distilled water. 4-aminonitrophenyl (24 mg) was added to the phenol solution, which was further diluted to a final volume of 30 ml with glass-distilled water. These reagents were prepared fresh daily.

After the completion of the CLS test, the supernatant of each tube was assayed for myeloperoxidase activity. Lysis from treatments with antibody alone and complement alone was considered as background in these experiments. Supernatant (0.1 ml) of each specimen from each tube was added in succession into a 3-ml cuvette containing phenol/4-aminonitrophenyl solution (1.4 ml), and 0.0017 M hydrogen peroxide (1.5 ml). Instantaneous changes in absorbance at 510 nm (at 25°C) were graphically recorded for 4-5 min. The reaction rate is directly proportional to the slope (ΔA₅₁₀/time) derived from the linear portion of the curve. The slope is in turn directly proportional to the MPO activity. Enzyme activity (mg enzyme/ml reaction mixture) can be calculated by constructing a standard curve utilizing regular increments of horse radish peroxidase (Worthington Peroxidase D). For our purposes, it was only important to consider the relative myeloperoxidase content of each tube, rather than calculate the actual enzyme activity. The reference point for maximal lysis (100% lysis) was calculated individually for each experiment, was considered to be that quantity of MPO released in the test tube containing cells, antibody, and complement at 1:1 dilutions. Triton X-100 or other nonionic detergents interfered with the colorimetric assay and could not be used to obtain maximal lysis. Percent lysis was calculated according to the formula:

\[
\text{Percent cell lysis} = \frac{(\Delta A_{510}/\text{time}) \times 1/n \times \text{dilution}}{(\Delta A_{510}/\text{time}) \times 1/1 \times \text{dilution}} \times 100\%
\]

where 1:n is the serial complement dilution.

**Marrow Culture Technique**

Buffy coat cells were obtained as in myeloid precursor studies and resuspended in 5 ml of alpha-media. The cell suspension was layered atop a Ficoll-diatrizoate gradient (LSM solution, Bionetics, Kensington, Md.) and centrifuged at 500 g for 10 min and the supernatant fluid removed. Cells were resuspended and washed with alpha-media. Quadruplicate cultures were plated in 35 x 10 mm Petri dishes with 2-mm grid (Lux, Newburg Park, Calif.) using a cell concentration of 2 x 10⁶ cells/ml/dish. Each dish contained 0.8% methylcellulose (Fisher Scientific Co., Fairlawn, N.J.) and 20% fetal calf serum containing undetectable amounts of complement (GIBCO, Grand Island, N.Y.) in alpha-medium (containing 100 U/ml of penicillin and 100 mg/ml streptomycin). Ten percent human placental conditioned medium was used as the source of myeloid colony-stimulating factor (CSF). The myeloid CFU-c cultures were incubated in a humidified, 37°C, 7.5% CO₂ incubator. The cultures were periodically checked and scored after 14 days of incubation (day of maximum CFU-c growth in our laboratory), recorded as colonies per 2 x 10⁶
cells cultured. Aggregates of ≥ 50 cells are defined as representing a CFU-c colony.

RESULTS

Erythroid Cell Precursor Studies
$^{59}$Fe as a Suitable Radioactive Label

The specific incorporation of $^{59}$Fe by erythroid precursors was demonstrated by autoradiography of marrow cells from normal patients. Further, we also demonstrated that $^{59}$Fe release was incorporated into heme by the erythroid precursor cells extractable by cyclohexanone. In selected experiments, after the CLS test, heme was extracted from both surviving cells with cyclohexanone and the supernatant fluid after lysis. $^{59}$Fe was found in heme from both components.

Purification of Erythroid Precursor Cells

Since both PNH$^{37}$ and non-PNH$^{37,38}$ reticulocytes have been shown to incorporate $^{59}$Fe during hemoglobin synthesis, it was important to make the bone marrow sample as "reticulocyte free" as possible. Therefore, a hypotonic lysis procedure was used. After lysis, less than 1% of the cells remaining were non-nucleated and less than 5% of the radioactivity could be accounted for by these non-nucleated cells. Thus, the radioactivity measured after the hypotonic lysis procedure was almost entirely in the nucleated erythroid precursors. The autoradiographs of the bone marrow preparation after lysis also confirmed that the radioactivity was in the nucleated erythroid precursors and not in other marrow cells. The autoradiography results were in agreement with the finding of others.$^{39,41}$

Lysis of $^{59}$Fe-Containing Marrow Cells by Anti-I Antibody and Complement

With variable dilutions of complement added, cytolysis, as determined by $^{59}$Fe release, was greater from marrow of patients with PNH as compared to the marrow of non-PNH individuals (Fig. 1). The maximum release of $^{59}$Fe from non-PNH erythroid precursors ranged from 6% to 14%, while that from PNH precursor cells ranged from 22% to 60% of total $^{59}$Fe radioactivity. The amount of complement required to attain 50% of the maximal release of $^{59}$Fe of PNH cells (1/20 ± 0.28/20 SEM) was less than that required for non-PNH cells (1/2 ± 0.26/3 SEM) (Table 2).

Myeloid Precursor Studies

Purification of the Myeloid Precursor Cells

Since peripheral granulocytes contain significant amounts of MPO, it was imperative to obtain as pure a preparation of myeloid precursor cells (before myelocyte stage) as possible. Representative cell layers separated on the BSA gradient and subsequently utilized in the CLS test, were stained with Wright's solution (Fisher Scientific Co., Fairlawn, N.J.) to determine the morphology of those cells studied. The composition of the PNH test preparations was approximately 40%--50% erythroid precursors, 20%--25% small unidentifiable mononuclear cells, 15%--20% myeloid precursors, 0%--5% monocytes, 0%--5% basophils, <1% PMNs.

Complement Sensitivity of Myeloid Precursor Cells by Antibody and Complement

We have demonstrated that MPO content correlates linearly with the number of myeloid precursor

Table 2.

<table>
<thead>
<tr>
<th>Type of Study</th>
<th>Patient</th>
<th>Marrow Cellularity</th>
<th>C'Dilution for 50% Lysis</th>
</tr>
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<tbody>
<tr>
<td>Erythroid*</td>
<td>1</td>
<td>Increased</td>
<td>1/40</td>
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<tr>
<td></td>
<td>2</td>
<td>Increased</td>
<td>1/20</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>4</td>
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<td>1/15</td>
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<tr>
<td>Myeloid†</td>
<td>5</td>
<td>Increased</td>
<td>1/43</td>
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<tr>
<td></td>
<td>6</td>
<td>Increased</td>
<td>1/20</td>
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<td></td>
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<td>1/5</td>
</tr>
<tr>
<td></td>
<td>8</td>
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<td>1/10</td>
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<tr>
<td></td>
<td>1A</td>
<td>Increased</td>
<td>1/11.5</td>
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<tr>
<td>Marrow culture‡</td>
<td>9</td>
<td>Increased</td>
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<tr>
<td></td>
<td>10</td>
<td>Increased</td>
<td>Not done</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Increased</td>
<td>Not done</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Normal</td>
<td>Not done</td>
</tr>
</tbody>
</table>

* Normal range of 50% C'-induced erythroid lysis 1/3 ± 0.26/3 SEM (n = 4).
† Normal range of 50% C'-induced myeloid lysis 1/5.5 ± 0.14/5.5 SEM (n = 10).
‡ Does not include patients already mentioned in table.
cells lysed by sonication (Fig. 2). This relationship can be expressed by:

\[(\Delta A_{510}/\text{time}) = K \times \text{number of cells lysed by sonication}\]

Thus, MPO release in the supernatant fluid can be used as an indicator of the relative number of cells lysed in the CLS test.

The dilution of complement yielding 50% of maximum lysis was considered to be representative of the sensitivity of the myeloid precursor cell population. Ten non-PNH marrow specimens required complement dilutions between 1/5 and 1/6 to achieve 50% of the maximum lysis. Two PNH marrows from patients 5 and 6 demonstrated a cell population markedly sensitive to complement, requiring less of it in dilutions of 1/43 and 1/20 (results obtained by computation), respectively, to attain a similar level of 50% maximum lysis. Patients 1A and 8 exhibited intermediate sensitivity to complement lysis, requiring less of it in dilutions of 1/1.5 and 1/10, respectively. The lysis curve of PNH patient 7 appears to conform closely to the non-PNH lysis curve, requiring a complement dilution of 1/5.5 (see Discussion) (Figs. 3 and 4, Table 2).

**CFU-c Culture Studies**

All PNH patients demonstrated poor CFU-c growth in tissue culture when compared to normal controls (Table 3). The marrow of 4 of 7 PNH

<table>
<thead>
<tr>
<th>Patient</th>
<th>2 x 10^5 Colonies/2 x 10^5 Cells Plated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td>NG</td>
</tr>
<tr>
<td>8</td>
<td>NG</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>NG</td>
</tr>
<tr>
<td>11</td>
<td>NG</td>
</tr>
<tr>
<td>12</td>
<td>26</td>
</tr>
</tbody>
</table>

NG, no growth.

*Normal average—40 colonies ± 15 SEM.
patients demonstrated no CFU-c growth and the other 3 PNH marrows exhibited suboptimal growth characteristics (normal marrow: 40 ± 15 SEM colonies/2 x 10^5 cells plated). The colonies were similarly composed of myeloblasts and granulated precursors. Attempts to stain cultured cells for LAP in order to examine the possibility of clonal differences failed in PNH marrows as well as normal marrows.

**DISCUSSION**

The objective of this investigation was to determine whether the previously reported increased susceptibility of PNH peripheral red cells, white cells, and platelets to complement-mediated lysis is also a characteristic of erythroid and myeloid precursors in the bone marrow. In order to examine this problem, ^59^Fe and myeloperoxidase were used as specific markers to monitor the respective lytic action of complement and antibody on erythroid and myeloid cell precursors. The validity of ^59^Fe release as a measurement of liberated hemoglobin from lysed erythroid precursor cells has been substantiated by Krantz et al. The use of MPO release as a marker of myeloid precursor cell lysis has not been previously reported. The commonly used method of labeling the cells with radioisotopes, such as ^51^Cr or DF32P, is nonspecific and would require isolation of the desired cell population first. The MPO assay proved to be feasible and highly reliable for the detection of myeloid cell lysis. The RBCs, lymphocytes, plasma cells, and macrophages contain non-detectable MPO by this method and thus do not interfere with the specificity of the assay for myeloid cells. It may be applicable for the in vitro study of a variety of clinical conditions in which increased myeloid cell destruction is suspected (e.g., immune neutropenia due to antibody-dependent cytotoxicity, drug-induced neutrophil destruction, etc.).

Erythroid cell precursors from patients with PNH are more susceptible to the hemolytic action of complement than the precursors of normal donors. This indicates that the membrane defect is present in the committed, hemoglobin-producing erythroid precursor cells during erythropoiesis, before they reach final maturation.

Myeloid cell precursors of two PNH patients demonstrated significantly greater sensitivity to complement lysis than did non-PNH precursor cells. It is interesting to note that these two patients were in hemolytic crisis at the time their marrows were obtained. Two other patients demonstrated intermediate complement sensitivity. Both of these patients were experiencing morning hemoglobinuria and were clinically evaluated as being in a mild phase of their disease. Patient 7, whose marrow myeloid lysis curve was near the normal range, had only 13% abnormal red cells. The proportion of abnormal precursors in this patient in our study may have been too small to be detected by this technique. Alternatively, but less likely, his marrow may lack those abnormal cells. Demonstration of increased complement sensitivity in the erythroid and myeloid precursor cells suggests that the marrow cells may not be protected from the lytic event of PNH. However, it is our frequent observation that compensating marrow hyperplasia occurs with hemolytic episodes in PNH patients. This observation may imply possible compartmentalization between the marrow and the blood in terms of the exposure to inciting factor(s) and the subsequent lysis. Similar observations have been made in patients with intravascular immune destruction of red cells, white cells, or platelets.

Poor marrow CFU-c growth in PNH has been previously reported. Marrows of our PNH patients generally demonstrate reduced colony numbers in tissue culture, thereby implicating either a proliferative abnormality or the lack of response to colony-stimulating factor in vitro. At present, we do not know whether the CFU-c found in PNH marrows represent the normal population or a combined growth in subnormal numbers. An attempt to distinguish cell populations of LAP levels failed because the cultured cells could not be stained for alkaline phosphatase.

In light of the increased complement sensitivity of all three mature blood elements, erythroid and myeloid precursor cells, and the poor CFU-c growth in PNH similar to that seen in myelodysplasia and leukemia, the defect in PNH may occur in the early progenitor cells. These studies do not delineate the earliest hematopoietic cell that demonstrates the defect. Since both red cell and granulocyte precursors demonstrate increased complement sensitivity, the defect may be presumed to be present in a cell species preceding both—the stem cell.

**ACKNOWLEDGMENT**

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J Tumen, LB Kline, JW Fay, DC Scullin, EG Reisner, WF Rosse and AT Huang

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