Two Populations of Granulocytes in Paroxysmal Nocturnal Hemoglobinuria

By M. Stern and W. F. Rosse

The granulocytes in paroxysmal nocturnal hemoglobinuria (PNH) are defective, and the defect is similar to that previously described for the PNH erythrocyte. Using anti-I antibody to activate complement and $^{51}$Cr release to detect cell lysis, we found two populations of granulocytes that differed in their susceptibility to lysis by complement in 5 of 6 patients. A proportion of the cells were lysed by one-fifteenth to one-twentieth the amount of complement required to lyse normal cells; the remainder of the granulocytes appeared to be normal in their susceptibility to the lytic action of complement. The binding of the third component of complement (C3) to PNH granulocytes was at least twice that bound to normal cells, even though the binding of antibody was the same for normal and PNH cells. This suggests that the binding of C3 and probably the efficiency of the terminal steps of complement lysis are increased in the abnormal PNH granulocyte. These defects affect only a portion of the granulocytes, thus suggesting that the disorder is a clonal stem cell abnormality.

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) is a complex disease characterized by erythrocytes, platelets, and granulocytes that are abnormal in their interactions with complement. The red cell has been most thoroughly investigated. In most patients, two (occasionally three) populations of red cells exist that differ in their susceptibility to the lytic action of complement. Some red cells (PNH I cells) appear to be normal in that they require large amounts of complement for lysis. A few patients have a population of moderately abnormal red cells (PNH II cells) that require about one-fifth as much complement as normal cells for lysis. Most patients have some markedly abnormal red cells (PNH III cells) that require about one-twentieth the amount of complement as normal cells for lysis. The proportions of the populations vary greatly from patient to patient. The abnormal susceptibility to complement lysis appears to be due to two defects. All abnormal PNH red cells fix three to five times as much of the third component of complement (C3) as normal cells, although the fixation of antibody, C1, C4, and C2 is identical on all cells. Further, the terminal steps of complement are markedly more efficient in the PNH III red cells, since more lysis occurs for a given amount of C3 fixation. This appears to be due to the fact that fewer terminal complexes must be assembled in order for successful penetration of the membrane to take place.

Platelets and granulocytes are also abnormal in PNH. Thrombocytopenia and neutropenia occur frequently in patients with the disorder. Platelets from PNH...
patients are 10–30 times more sensitive to the lytic action of complement than normal platelets.\(^5\) Further, PNH platelets fix more C3 for a given amount of activation of complement by antibody, and this membrane-bound C3 causes the platelet release reaction to occur. These events are thought to account, at least in part, for the increased incidence of venous thrombosis in the disorder.\(^6\)

PNH granulocytes are also more susceptible to the lytic action of complement than normal.\(^5,7\) There also appears to be impaired phagocytosis\(^8\) and chemotaxis\(^9\) in the presence of activated complement. It has also been shown that the mean leukocyte alkaline phosphatase of granulocytes in patients with PNH is low or absent, as measured by cytochemical staining.\(^10\) Those granulocytes that, after treatment with activated complement, migrate normally in the presence of chemotactic factors have normal amounts of leukocyte alkaline phosphatase, whereas those cells that demonstrate impaired chemotaxis have subnormal levels of leukocyte alkaline phosphatase.\(^7\) This is taken as evidence that more than one population of granulocytes exist.

We have studied PNH granulocytes to characterize further the membrane defect responsible for the granulocyte abnormalities that have been observed in the disorder. We have found that a proportion of PNH granulocytes bear membrane abnormalities similar to the defects of the red cell and the platelet. This is further evidence that PNH is a clonal stem cell disorder in which all of the blood elements are affected in the same way.

**MATERIALS AND METHODS**

**Buffers**

Veronal-buffered saline (VBS) was used throughout, unless otherwise noted, and was made according to Mayer.\(^11\) Modified Alsever’s solution was used as an anticoagulant and was made according to Mayer.\(^11\)

**Serum**

Normal serum was obtained by venesection into cooled plastic cups that were immediately centrifuged. The plasma was removed and allowed to clot at room temperature.

Serum from a patient with cold agglutinin disease (Stept.) was kindly donated by Dr. John Crookston, Toronto General Hospital. The serum was frozen at −90°F until required for use. Cold agglutinins were purified and labeled with \(^{125}\)I according to methods described previously.\(^3\)

**Granulocyte Preparation**

Granulocytes from normal donors and PNH patients, none of whom were infected or leukocytotic, were prepared from leukocyte-rich plasma obtained by sedimentation at 1 g of 50–100 ml of heparinized blood in a plastic syringe for 2–3 hr. Sedimenting agents such as plasmagel and dextran were not used.

The leukocyte-rich plasma was layered on Ficoll-Hypaque (specific gravity 1.080) and centrifuged at 1000 g for 20 min. The granulocyte-containing fraction at the bottom of the tube was washed once in Alsever’s solution, and contaminating erythrocytes were lysed by a 5-min incubation in a 37°C water bath with Tris-buffered 0.83% ammonium chloride, pH 7.2. The granulocytes were then washed and resuspended in 2 ml of Alsever’s solution. The resulting concentration of granulocytes was approximately 10⁶ cells/ml.

**Determination of Complement-Mediated Lysis**

Three hundred microcuries of sodium chromate (\(^{51}\)Cr) of high specific activity were added to the 2-ml suspension of granulocytes, and the mixture was incubated in a 37°C water bath for 1 hr. The granulocytes were then washed three times with Alsever’s solution and adjusted to a concentration of
5 × 10⁶ cells/ml. To each chamber of a Microtiter plate were added 0.05 ml of chromated granulocytes, 0.05 ml of cold agglutinin antibody, and 0.05 ml of human serum in varying dilutions (1:1 to 1:81). The plate was placed in an ice bath for 30 min and then incubated in a CO₂ incubator at 37°C for 1 hr. The Microtiter plate was then centrifuged at 1000 g for 20 min, and the supernatant fluid of each chamber was decanted for determination of released radioactivity as a measure of granulocyte lysis. The amount of ⁵¹Cr released on complete lysis was measured by lysing the granulocytes with 1% desoxycholate (DOC). The amount of ⁵¹Cr released when complement was omitted was also determined.

**Determination of Membrane-Bound C₃**

The concentration of granulocytes was adjusted to 5 × 10⁷ cells/ml in approximately 0.4 ml of Alsever’s solution. C₃ was fixed by mixing 0.1 ml of granulocytes with 0.1 ml of human serum and 0.2 ml of diluted serum containing the cold agglutinin antibody or 0.2 ml of purified cold agglutinin. Protein concentration was determined by the Lowry method, and the purified antibody (labeled or unlabeled) was adjusted to a concentration of 0.1 mg/ml. The mixture of granulocytes, serum, and antibody was placed in an ice bath for 30 min and then warmed to 37°C in a water bath. Five milliliters of Alsever’s solution at 37°C were added to the samples before centrifuging for 10 min at 12,000 g. Five milliliters of VBS were added to the C₃-coated granulocyte pellet, and the samples were again centrifuged for 10 min at 12,000 g.

The quantity of C₃ on the membranes of granulocytes was determined by the anti-C₃ absorption technique of Borsos and Leonard as modified by Logue et al. The C₃-coated granulocytes were incubated with a standard amount of anti-C₃ capable of lysing 60%-80% of sheep cells coated with human complement components (E⁹C₄₃⁹⁺) after the addition of guinea pig complement. The absorption of anti-C₃ from solution was detected by the decrease in lysis of the C₃-coated sheep cells (E⁵⁸C₄₃⁹⁺). By comparing the amount of anti-C₃ remaining after absorption with the C₃-coated granulocytes to a standard curve obtained with known amounts of purified C₃, the amount of C₃ on the granulocyte membrane could be determined and expressed as molecules of C₃/cell.

To determine the amount of C₃ binding to the granulocyte per molecule of antibody adsorbed, several dilutions of purified ¹²⁵I-labeled cold agglutinin antibody were incubated at 0°C for 30 min with the sample granulocytes and then centrifuged at 1000 g for 10 min to determine the amount of radioactivity adsorbed to the cells.

**RESULTS**

The results of lysis of granulocytes by antibody and complement are shown in Fig. 1. Normal cells appear to require large amounts of complement for lysis.
TWO POPULATIONS OF GRANULOCYTES IN PNH

Although a single population appeared to be present, the amount of lysis was insufficient to determine this with certainty. In 5 of the 6 patients with PNH, two populations of granulocytes were found, one markedly more sensitive to complement than the other, which appeared to be normal. The proportion of abnormal cells could be determined by the inflection point of the curve, as for studies of the red cell. The sensitive cells in these patients constituted between 6.1% and 12.3% of the total granulocyte population in 4 patients and 42.9% in the fifth. In most instances the proportion of abnormal granulocytes was less than the proportion of markedly abnormal red cells (Fig. 2, Table 1). One patient had a very small proportion of markedly abnormal red cells, and no abnormal granulocytes were detected.

The relative sensitivities of the populations of granulocytes may be determined by calculating the dilution of complement that would lyse 50% of the cells. The reciprocal of this dilution is the so-called complement lysis sensitivity (CLS) titer. In the case of the abnormal granulocytes in PNH, the dilution of serum yielding 50% lysis is about 1:15. In normal cells or normal-like PNH cells, 50% lysis was not obtained even in undiluted serum. Therefore, the measure of CLS must be derived by extrapolation. The CLS titers were compared for all patients with PNH; the abnormal cells from different patients were similar (Table 1).

The adsorption of an anti-I antibody and the fixation of C3 to PNH granulocytes were measured. Although the mean adsorption of antibody to PNH granulocytes

<table>
<thead>
<tr>
<th>Patient</th>
<th>Percentage Abnormal Granulocytes</th>
<th>Percentage PNH III Red Cells</th>
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<tr>
<td></td>
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<tr>
<td>Normal controls</td>
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*Values in parentheses derived by extrapolation.
was less than the mean adsorbed to normal cells, there was no statistical difference in the amount of antibody adsorbed under similar conditions (Fig. 3). More C3 was fixed to PNH granulocytes than to normal granulocytes. Thus, for a given amount of antibody fixed, more C3 is fixed to PNH than to normal granulocytes (Fig. 4).

**DISCUSSION**

In these studies on granulocytes we have been able to demonstrate, in 5 of 6 patients, two populations that differ in their susceptibility to the lytic action of complement, as has recently been shown in a single patient. This suggests that the granulocyte defect is present in a clone of cells that constitutes only a portion of the
circulating myeloid cells. Since a portion of red cells in such patients are similarly abnormal, it is likely that these abnormal granulocytes and red cells are derived from a clone of abnormal stem cells.

The proportion of abnormal circulating granulocytes in a patient appears to be less than the proportion of abnormal erythrocytes in the same patient in most cases. The assessment of lysis of granulocytes by measuring the release of $^{51}$Cr may underestimate the degree of lysis by antibody and complement and thus underestimate the proportion of abnormal cells, since the maximal release of radioactivity with DOC may exceed the maximum possible lysis by immunologic processes. However, others have used such measures in assessment of immune lysis of granulocytes.\textsuperscript{14}

We have previously shown that the proportion of abnormal erythroid precursors in PNH exceeds the proportion of abnormal circulating cells,\textsuperscript{15} indicating that the proportion of PNH stem cells is even greater than the proportion of abnormal circulating red cells. The discrepancy is accounted for by the increased rate of hemolysis of the abnormal cells compared to the more normal cells. If granulocytes are derived from the same stem cell clone, then there is an even greater discrepancy between the number of stem cells and the number of circulating product cells. This suggests that the great majority of abnormal myeloid precursors may be destroyed before or shortly after entering the circulating blood pool. Alternatively, abnormal granulocytes might become marginated in the peripheral circulation and hence not be present when blood is drawn routinely. Fehr and Jacob have suggested that the fixation of complement to normal cells causes them to marginate.\textsuperscript{16} Studies of granulocyte kinetics in PNH have not shown abnormalities in total circulating life span,\textsuperscript{7} but the life span of the abnormal population may not be measured in these studies, since the circulating population may be small and the time in the circulation may be too short to measure accurately.

We were unable to detect more than one granulocyte population in one of our PNH patients who had a small proportion of PNH III cells and a large proportion of PNH II cells. This may reflect our inability to detect granulocytes corresponding to the "moderately" sensitive PNH II cells. Perhaps lysis by immunologic processes can only be demonstrated in the markedly sensitive granulocytes corresponding to PNH III red cells, and we, therefore, are able to detect two granulocyte populations only if a sufficient proportion of the markedly abnormal cells is in the peripheral circulation.

The interaction of complement with all three cell lines (red cells, platelets, and granulocytes) results in abnormally increased fixation of C3. For the granulocyte, this may account for the abnormalities in chemotaxis that are seen after exposure to activated complement.\textsuperscript{9}

These data were obtained on mixtures of normal-like and abnormal granulocytes, as it is not possible to separate out the abnormal populations of granulocytes for specific analysis. We cannot, therefore, measure the amount of C3 on the markedly abnormal cells alone. Although the CLS data suggest that more lysis occurs for a given amount of C3 fixed to granulocytes, we cannot affirm an increased efficiency of the terminal steps on the granulocyte as is seen on the PNH III red cell, since we cannot measure directly the amount of C3 on the abnormal cells.
In summary, our studies demonstrate that the granulocytes in PNH have abnormalities similar to those previously demonstrated for the red cell and platelet and that these abnormalities are present on only part of the granulocytes. This strongly suggests that PNH is a disorder of proportions of the stem cells that propagate abnormal cells in all three cell lines. These abnormal product cells have defective membranes that interact abnormally with complement in a consistent manner.

ACKNOWLEDGMENT
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