Neutrophil Life Span in Paroxysmal Nocturnal Hemoglobinuria

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We have studied neutrophil intravascular life span in six patients with paroxysmal nocturnal hemoglobinuria (PNH); four had normal neutrophil counts when studied and two were neutropenic. Five patients had enough circulating neutrophils to isolate for tests in vitro. Lysis of labeled neutrophils was greatly increased, compared to that of normal volunteers, when these neutrophils were incubated with acidified fresh serum as a source of active complement plus serum containing antineutrophil antibodies (from three different sources). Despite the in vitro lesion, however, each of these patients had a normal neutrophil intravascular life span as measured by the ^{32}P-diisopropylfluorophosphate technique. One neutropenic patient, who had a normal neutrophil life span, had a shift of cells from the circulating to marginated pool of sufficient degree to cause the neutropenia. A second (severely) neutropenic patient was found to have developed extreme marrow hypoplasia, also explaining the neutropenia. Thus, in contrast to the shortened red cell life span, we have been unable to find a shortened neutrophil life span in PNH.

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) is characterized in vitro by a marked lytic sensitivity of the patient's red cells to complement. This phenomenon may be responsible for the shortened life span of the red cells. A similar increased lytic sensitivity to complement has been demonstrated in vitro for neutrophils and platelets.[1] Since PNH patients are sometimes neutropenic, we have investigated the possibility that PNH patients have a shortened intravascular life span.

MATERIALS AND METHODS

Patient Selection

We have studied a total of six patients with PNH. Some selected features of these patients are shown in Table 1. Each patient exhibited features of intravascular hemolysis (hemoglobinemia, morning hemoglobinuria with clearing as the day progressed, absent serum haptoglobin, hemosiderinuria, and increased indirect reacting bilirubinemia). Each patient consistently showed a positive Ham test,[2] thrombin test,[3] sucrose lysis test,[4] inulin test,[5] and the four-tube complement lysis sensitivity test using high-titer cold-agglutinin sera as the antibody to sensitize the red cells.[6] Their red cells had a low level of activity of acetylcholinesterase,[7] and their neutrophils had low alkaline phosphatase activity.[8]

Abbreviations used in this paper: PNH, paroxysmal nocturnal hemoglobinuria; $t_{\frac{1}{2}}$, 50% clearance time; DF^{32}P, ^{32}P-diisopropylfluorophosphate.

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Submitted July 28, 1976; accepted May 13, 1977.

Supported by the Medical Research Service of the Veterans Administration, Grant 5 R01 CA 11929 from the National Cancer Institute, and Grant RR-287 from the General Clinical Research Centers Program of the Division of Research Resources, NIH.

Presented in part at the Meeting of the Southern Section, American Federation for Clinical Research, New Orleans, La., January 1971.

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 ISSN 0006-4971.

Table 1. Data on PNH Patients in the Study

Patient No.	Age (yr), Sex	Neutrophil Count* (cells/cu mm)	History of Aplastic Anemia	Comments
1	30,F	4000–5700	Before PNH	Previously splenectomized and never neutropenic with PNH
2	54,M	2900	No	Never neutropenic with PNH
3	45,M	3000	No	Never neutropenic with PNH
4	50,M	2600	No	Never neutropenic with PNH
5	15,F	700	Developing aplasia	Falling neutrophil count
6	34,F	1300	Before PNH	Neutropenic since aplasia

*Normal range: 1830–7250 cells/cu mm.[17]

Neutrophil Lysis In Vitro

Each patient (except patient 5, who had too few neutrophils to study) was phlebotomized of 500 ml blood into acid-citrate-dextrose USP Formula A anticoagulant, after giving appropriate informed consent. This blood was centrifuged in a refrigerated centrifuge at 1500 *g* for 3 min. The blood bag was carefully removed from the centrifuge, the plasma was removed, and the buffy-coat layer and the upper layer of red cells were carefully squeezed into a satellite bag. This buffy coat was mixed with an equal volume of 3% dextran (molecular weight 200,000), 0.45% saline, and 2.5% dextrose and allowed to settle. The white cell-rich upper layer of dextran-plasma was removed in approximately 30 min, centrifuged, and subjected to hypotonic lysis[9] in order to remove the remaining few red cells. ^{32}P-Diisopropylfluorophosphate (DF^{32}P; PB118P, Amersham/Searle Co., Arlington Heights, Ill.) 5–20 μCi in 0.1 ml volume was added to the white cells contained in about 1 ml of 0.9% saline. This suspension was incubated at room temperature for 45 min. The white cells were then washed three times by resuspending in saline and centrifuging, then removing the supernatant saline containing the unattached isotope. At this point the white cell suspension consisted of approximately 90% neutrophils, 10% mononuclear cells, essentially no red cells, and only occasional platelets. The viability of the white cells was demonstrated by their ability to exclude trypan blue dye.

Simultaneously, fresh human serum was prepared from normal donors and adjusted to pH 6.6 (found to be the optimum pH) with HCl. Half of this serum was incubated at 56°C for 30 min, and the other half was kept in an ice bath.

Neutrophil antibody serum was obtained from various sources. One was a volunteer who had been immunized with allogenic white cells (kindly donated by Dr. Henry Wilson, Ohio State University Medical Center, Columbus, Ohio). Another was a patient with high-titer red cell cold agglutinins. Three of nine such patients in our experience had serum antineutrophil activity. Later, we used serum from a patient with cyclic neutropenia who had potent antineutrophil antibodies as demonstrated by the method of Boxer and Stossel.[10]

Incubation mixtures were then prepared containing 0.8 ml of serum, either fresh or fresh heat-treated, 0.1 ml of white cell suspension, and 0.1 ml of serum presumed to contain antineutrophil antibodies.

These mixtures were incubated at 37°C for 1 hr, except when the antiserum was from a patient with cold agglutinin disease. In the latter case, the mixture was preincubated at 0°C for 30 min followed by the 37°C incubation for 1 hr. Each tube was centrifuged at 1000 *g* for 10 min and the supernatant serum and cells were separated. The cells were solubilized with 1 ml of Soluene 100 (Packard Instrument Co., Downer's Grove, Ill.) and counted in a liquid scintillation spectrometer. Because of the high energy of ^{32}P, slight variations in quenching had little effect on the counts, so that no corrections were necessary. For each tube the percentage lysis was calculated by dividing the serum counts by the sum of serum and cell counts (× 100).

Omission of the antineutrophil antibody serum from the incubation mixture essentially eliminated the excess lysis seen when it was included with fresh serum and PNH white cells.

We verified that the PNH neutrophils were being lysed by incubation with fresh serum and antineutrophil antibody by examining the cells microscopically at the end of the incubation after adding trypan blue. The cells were noted to be degenerated and clumped, and nearly all took

up the stain. Normal neutrophils under the same conditions showed no such degeneration. PNH neutrophils similarly incubated with heat-inactivated fresh serum also showed no such degeneration, but appeared as normal neutrophils.

Measurement of Neutrophil Life Span

The $DF^{32}P$ neutrophil survival study was done by a slight modification of the procedure of Mauer et al.[11] as previously reported.[12] From this procedure we obtained sequential values for specific white cell radioactivity expressed as the percentage of the expected specific activity if the labeled blood had been diluted to the calculated blood volume.[13] The data were plotted in the usual fashion on a logarithmic scale against time on an arithmetic scale. The points so obtained were fitted to a straight line by standard computerized techniques and the theoretical line of best fit was utilized to calculate a $t_{\frac{1}{2}}$. The subjects gave appropriate informed consent for these studies.

RESULTS

Presence of In Vitro Lesion in PNH Neutrophils

In vitro neutrophil lysis is shown in Table 2. Neutrophils from PNH patients had a greater percentage lysis than neutrophils from normal controls when these cells were incubated with complement (fresh human serum) and serum containing antineutrophil antibodies. When complement was inactivated (by heating to 56°C for 30 min) or antineutrophil antibody serum was omitted, the lysis of PNH neutrophils was similar to that of normal neutrophils under the same circumstances.

Figure 1 shows that exposure of one of these patient's neutrophils to various dilutions of complement produced a pattern of lysis that demonstrated a double population of cells, one more sensitive and one less sensitive. This pat-

Table 2. Results From Both In Vitro and In Vivo Studies

Patient No.	Net Neutrophil Lysis (%) In Vitro*		Source of Antibody†	Neutrophil Count‡	In Vivo $DF^{32}P$ Neutrophil Turnover Data§				
	PNH	Normal		(cells/cu mm)	$t_{\frac{1}{2}}$(hr)	TBNP ($\times 10^7$/kg)	CNP ($\times 10^7$/kg)	MNP ($\times 10^7$/kg)	NTR ($\times 10^7$/kg/day)
1	26	8	a	4000	5.7	89	79	10	260
				5700	5.9	166	141	25	470
2	25	2	b	2900	8.2	106	46	60	220
3	14	2	b	3000	10.0	158	52	106	270
4	23	3	c	2600	8.0	146	40	106	300
5	Could not test	—	—	700	—	—	—	—	—
6	36	3	c	1300	6.5	47	11	36	120

*Percentage lysis (see Materials and Methods) of either PNH patient or normal control leukocytes (mostly neutrophils) incubated with fresh serum minus the percentage lysis (range 4%–16%) of the same cells incubated with heated (at 56°C for 30 min) fresh serum.

†a, serum from volunteer immunized against mixed human leukocytes (courtesy of Dr. Henry Wilson, Ohio State University Hospital); b, serum from patient with high-titer red cell cold agglutinins, also containing antineutrophil activity (see Materials and Methods); c, serum from patient with antineutrophil antibody demonstrated by method of Boxer and Stossel.[10]

‡Normal range: 1830–7250 cells/cu mm.[17]

§$t_{\frac{1}{2}}$, 50% survival time calculated from straight line of best fit for experimental points (normal 4–10 hr);[14] TBNP, total-body neutrophil pool (normal 27–138);[14] CNP, circulating neutrophil pool (normal 13–49);[14] MNP, marginated neutrophil pool (normal 0–85);[18] NTR, neutrophil turnover rate (normal 62–400).[14]

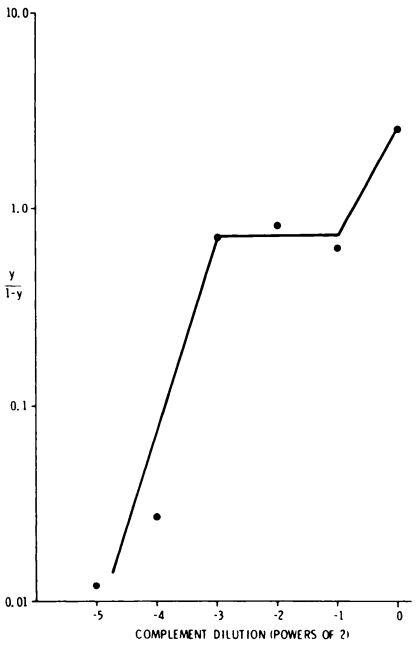

Fig. 1. Lysis by graded amounts of human complement of $DF^{32}P$-labeled leukocytes (mostly neutrophils) from PNH patient 4: Von Krogh logarithmic transformation plot (after Rosse and Davie[6]). y, fraction of cells lysed as calculated by the method of Aster and Enright,[1] in which release of 70% of the $DF^{32}P$ was taken as 100% immune lysis. Value of y/1 − y of 1.0 equals 50% lysis. Serial dilutions of fresh human serum (complement) were made into the same serum that had been heated (to 56°C for 30 min). Both fresh and inactivated serum were adjusted to pH 6.6. Incubation mixtures were as follows: 0.8 ml serum (complement dilution), 0.1 ml labeled cells, and 0.1 ml rabbit anti-human white blood cell serum (heated to 56°C for 30 min). Incubation was for 1 hr at 37°C. In this type of plot, a single population of cells would be a straight line with positive slope. This curve suggests two populations whose lines are joined by a horizontal line at y/1 − y = 0.7. By solving for y, one can calculate that the more complement-sensitive population (the cells lysed by a complement dilution of 1/8 or less) constitutes about 30% of the total.

tern is characteristic of red cells in the disease.[6] The percentage of more sensitive white cells was approximately 30%, similar to the approximately 25% sensitive red cells in the same patient as tested by Rosse and Dacie's method.[6]

Results In Vivo

The calculated $t\frac{1}{2}$ values for the $DF^{32}P$ neutrophil survival procedure in these patients is shown in Table 2; it ranged from 5.7 to 10.0 hr. These values fall within the 95% limits for normals as published by Bishop et al.[14] Our own values on seven normal controls and five otherwise hematologically normal patients with Hodgkin disease were 3.2–8.7 hr, calculated for the first 10 hr of the procedure.[15] Table 2 also gives values for the total-body, circulating, and marginated neutrophil pools and the neutrophil turnover rates[18] in these patients.

Results in Neutropenic Patients

Patients 5 and 6 were neutropenic at the time of the study. Patient 5 was so severely neutropenic that an adequate study could not be obtained. She had developed PNH without prior history of hematologic disease, but at the time we were able to study her she had developed aplastic anemia. The cellularity of her marrow biopsy was nearly zero and a differential count on the marrow smear was 91.5% lymphocytes, 2% total neutrophilic cells, and 2% total erythroid cells. (This sequence of events has been previously reported.[16]) Thus, her neutropenia was predominantly due to lack of marrow production. This pattern persisted until her death from fulminating sepsis 3 mo after we had studied her.

Patient 6 was reported to have had aplastic anemia prior to developing PNH. At the time of our examination her bone marrow was 70% cellular with a myeloid to erythroid ratio of 0.3:1. Although her neutrophil count (1300/cu mm) was well below normal limits, her $DF^{32}P$ study (Table 2) showed a normal $t_{\frac{1}{2}}$ and neutrophil turnover rate. The circulating neutrophil pool was below normal, but the marginated neutrophil pool was well within normal limits. Thus, her mild neutropenia was an example of "shift" neutropenia (shift from the circulating into the marginated pool), as defined by Bishop et al.[14] She definitely did not have a shortened neutrophil life span, in contrast to her short red cell life span [evidenced by marked hemoglobinuria, hematocrit of 29%, reticulocyte count of 8.5%, doubly corrected reticulocyte production index of 3 (within the range for hemolytic anemia[19]), and average yearly transfusion requirement of 12 units].

DISCUSSION

These results indicate that patients with PNH have a membrane defect of their neutrophils that can be consistently demonstrated in vitro, as previously reported.[1] This defect does not appear to be expressed in vivo, however. We were unable to demonstrate a shortened neutrophil life span in PNH patients whether neutropenic or not. Similarly, Aster and Enright[1] demonstrated that the in vitro defect of PNH platelets is not accompanied by a shortened life span.

Both neutropenia and thrombocytopenia are commonly observed during the course of PNH, neutropenia being present at some time in about 60% of these patients.[20] Our observations in neutropenic patients 5 and 6 strengthen the previous impression that the cause of neutropenia in PNH may be related to a defect in production,[20] or at least is not related to a shortened life span.

Bacterial infections are also known to be frequent in patients with neutropenia due to deficient production. Patient 5 died of an acute, fulminating sepsis similar to other patients with severe neutropenia due to marrow production failure. It is possible that the lesion demonstrated in vitro in PNH neutrophils may also contribute to this susceptibility to bacterial infection in that the neutrophils may lyse at a time when they should be phagocytosing and killing bacteria even if they have survived normally up to that point.

Aster and Enright[1] did not claim to have found a double population of platelets in PNH patients, one more and one less sensitive to the effects of complement and antibody, in contrast to the findings with PNH erythrocytes.[6] Their white cell studies, however, did suggest a "sensitive" population consisting of 80% 90% of the total ^{51}Cr-labeled cells, the rest being an insensitive population. They attributed this finding to lymphocytes in the mixed white cell preparation which are known to label well with ^{51}Cr.[21] We used $DF^{32}P$, which is predominantly a neutrophil label,[22] and also demonstrated a double population of cells (Fig. 1), thereby strengthening the evidence that the fundamental marrow lesion in PNH is a double population of undifferentiated stem cells, one giving rise to circulating blood cells with a membrane defect predisposing these cells to complement-mediated lysis and the other giving rise to more nearly normal circulating blood cells.

ACKNOWLEDGMENT

The authors wish to thank Chi-Hsien Teng, Charles Johnson, and Janet Paden for expert technical assistance. We are also indebted to Dr. Fernando Padilla, Dr. Samuel Poole, Dr. Edward Burka, Dr. David Martz, and Dr. William Cassel for permitting us to study their patients.

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