

## Complement-mediated Granulocyte Dysfunction in Paroxysmal Nocturnal Hemoglobinuria

By Philip R. Craddock, Jorg Fehr, and Harry S. Jacob

In paroxysmal nocturnal hemoglobinuria (PNH), infection, both viral and bacterial, disproportionate to the mild neutropenia seen in many such patients is responsible for significant morbidity. We report impaired granulocyte chemotaxis efficiency which may contribute to the problems induced by bacterial infections. PNH (but not normal) granulocytes, after exposure to very small concentrations of activated serum complement components, migrate poorly, as documented by their inhibited chemotaxis toward bacterial products or activated complement components in Boyden chambers. The granulocytes remain intact, excluding trypan blue, phagocytos-

ing, and killing bacteria, despite this activated complement exposure. It is also suggested that this chemotactic defect may involve only a clone of cells, analogous to the clonal lysis of PNH erythrocytes; those few granulocytes capable of migration after exposure to activated complement contain normal quantities of leukocyte alkaline phosphatase (LAP), in contrast to the LAP deficiency of the overall PNH granulocyte population. Since bacterial infection may initiate or potentiate hemolysis, one of the major symptoms of the disease, these results could explain much of the morbidity of PNH.

**I**N PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH), the erythrocytes, platelets, and neutrophils all appear to be abnormal in their interaction with activated components of complement.<sup>1-3</sup> It is clear that this unusual interaction brings about the destruction of erythrocytes in vivo, thus causing anemia; it is less clear that the abnormality, which is demonstrable in vitro, causes premature destruction of platelets or granulocytes in vivo, or accounts for the thrombocytopenia or leukopenia seen in these patients.<sup>4,5</sup>

Patients with PNH often develop severe bacterial disease; in one group of 52 patients, death in 7 was ascribed to such infections.<sup>6</sup> This propensity for infection may be due either to the mild leukopenia seen in these patients,<sup>4,5</sup> which we doubt, or alternatively to granulocyte dysfunction. Indeed, we have recently demonstrated that normal granulocytes exposed to activated complement become excessively sticky in vitro, and they marginate in vivo.<sup>7</sup> Since the cells in PNH are known to be hypersusceptible to complement,<sup>1-3</sup> we wished to determine whether an exaggeration of these complement-mediated abnormalities of motility might occur in PNH granulocytes. We have found that, al-

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Abbreviations used in this paper: PNH, paroxysmal nocturnal hemoglobinuria; LAP, leukocyte alkaline phosphatase; HBSS, Hanks' balanced salt solution; HSA, human serum albumin; MPO, myeloperoxidase.

though untreated PNH granulocytes migrate normally toward various chemotaxins, their motility is markedly inhibited after incubation with extremely low concentrations of serum in which the complement cascade has been maximally activated. These low concentrations of activated serum have no effect on cell viability or other granulocyte functions. This finding might explain the previously reported poor migration of PNH granulocytes into inflammatory skin windows *in vivo*.<sup>5</sup> Moreover, we suggest that this chemotactic defect in PNH might predictably lead to a propensity for infection similar to that which occurs in various other syndromes of chemotaxis dysfunction, such as the lazy leukocyte syndrome,<sup>8</sup> the Chediak-Higashi syndrome,<sup>9</sup> rheumatoid arthritis,<sup>10</sup> diabetes mellitus,<sup>11</sup> and severe hypophosphatemia.<sup>12</sup> These results have been preliminarily reported elsewhere.<sup>13</sup>

## MATERIALS AND METHODS

### Patients

All patients manifested intermittent intravascular hemolysis consistent with PNH (Table 1). One patient, J.O'R. has had recurrent staphylococcal furunculosis, refractory to standard therapy. Diagnosis was confirmed by strongly positive sucrose hemolysis and acidified-serum tests.<sup>14</sup> Patients L.L. and H.R. were diagnosed and treated at Duke University Medical Center, and the remainder at the University of Minnesota Hospitals. The proportion of circulating erythrocytes possessing the clonal susceptibility to complement-mediated hemolysis was determined with serum and cobra venom factor.<sup>2</sup> All patients were in the steady state at the time of study. None was undergoing acute hemolytic episodes. Two patients, L.L. and L.M., were being treated with low-dose alternate-day prednisone, but care was taken to study their granulocytes on their off-steroid days, more than 24 hr after their last dose. In ancillary studies, granulocytes from other, non-PNH patients treated with similar regimes of steroid therapy, responded normally in our chemotaxis assays when cells were studied on their off-steroid days. Indeed, in all the experiments reported here no difference in results with steroid-treated PNH-cells, compared to other PNH cells, was apparent. There was no correlation between the size of the complement-sensitive peripheral erythrocyte population and the impairment of granulocyte chemotaxis. For example, patient H.R., who had the greatest proportion of complement-sensitive erythrocytes, manifested only intermediate chemotaxis dysfunction, while patient B.L., with a smaller proportion of sensitive red cells, had the most inefficient granulocytes following activated complement incubation. All control studies were performed on peripheral granulocytes from normal laboratory personnel.

### Granulocyte Preparation

Blood from these patients or normal laboratory personnel was anticoagulated with heparin (4 U/ml) in plastic containers. Leukocytes were separated by dextran sedimentation,<sup>15</sup> washed twice in Hanks' balanced salt solution (Microbiological Associates, Md.), and finally resuspended at a concentration of  $5-10 \times 10^6$ /ml in HBSS, containing 0.5 g/100 ml human serum albumin (Hyland/Travenol, Calif.)

**Table 1. Hematologic Data on the Patients With PNH Included in This Study**

Patient	Leukocyte Counts (cells/cu mm)		Complement-sensitive Erythrocyte Population (Per Cent of Total Circulating Cells)
	Total	Neutrophils	
L.L.	6730	4370	65
H.R.	5400	2590	85
B.L.	3500	875	22
J.O'R.	3400	1200	26
L.S.	4200	3360	15
L.M.	6500	4125	10

### *Chemotaxin Generation*

Three chemotaxins were used: (1) the supernatant from cultures of *Escherichia coli*,<sup>16</sup> (2) serum complement activated by immune complexes,<sup>17</sup> and (3) serum complement activated by cobra venom.<sup>18,19</sup> The methods of preparation were as follows:

A pure strain of *E. coli* was grown overnight in Medium 199 containing phenol red (Microbiological Associates), centrifuged, and sterilized through a 0.2  $\mu$  filter to remove bacteria; the cell-free supernatant was diluted to 5% (v/v) in HBSS containing 0.5 g/100 ml HSA just prior to use as a chemotaxin.

Immune complexes were prepared by adding optimal precipitating quantities of HSA to anti-HSA antibodies raised in goats.<sup>20</sup> Crystalloid human albumin (Miles Pentax Laboratories, Ind.) was dissolved in isotonic saline at a concentration of 5 mg/ml. Serial double dilutions were made in saline. To aliquots of this diluted albumin were added equal volumes of goat serum containing anti-HSA antibodies (Kallestad Laboratories, Minn.), and the mixture was kept at 4°C for 24 hr. Optimum precipitation was obtained with an albumin concentration of 0.625 mg/ml. Thus, to 14 ml of goat serum were added 14 ml of albumin at this concentration, and, following cold incubation, the immune complex precipitate was centrifuged out at 10,000 g for 15 min and washed five times in cold isotonic saline. Optimal chemotactic activity was obtained with a ratio of 200  $\mu$ g of immune complex protein incubated with 1 ml of fresh AB serum at 37°C for 30 min; this ratio was used in all subsequent experiments. The activated serum was diluted 10% (v/v) in HBSS just prior to addition to the Boyden chamber.

Complement components were also activated by exposure of fresh AB serum to *Naja naja* cobra venom. The lyophilized venom (Ross Allen Venom Laboratory, Fla.) was purified by Sephadex G-100 chromatography.<sup>2</sup> The protein peak, of molecular weight greater than 80,000 daltons,<sup>19</sup> capable of inducing complement-mediated hemolysis specifically in PNH erythrocytes, was concentrated with collodion bag filtration (Schleicher and Schuell, Inc., N.H.) to provide a solution of 500  $\mu$ g protein/ml. Chemotactic activity was generated in fresh AB serum by incubation of 1 ml of this cobra venom solution with 3 ml of serum at 37°C for 30 min. This incubation resulted in maximal activation of the complement cascade, producing almost complete depletion (99%) of total hemolytic complement in the serum. To prevent the possibility of any additional complement activation after the cobra venom incubation, the serum was then heated at 56°C for 30 min, prior to use as a chemotaxin or prior to the preincubation with the granulocytes. Optimal chemotactic activity was obtained in the Boyden chamber with a 5.0  $\mu$ l/ml dilution of the cobra venom activated serum in HBSS.

### *Chemotaxis*

The granulocyte chemotactic response was measured *in vitro* by a modification of the method of Boyden.<sup>12,17</sup> To the upper compartment of a plastic Boyden chamber were added 0.2 ml of leukocyte suspension, and to the lower compartment 1.6 ml of the respective chemotaxins. The two compartments were separated by a Millipore filter with a mean pore size of 3  $\mu$ . The apparatus was placed in a humidified incubator at 37°C for 3 hr, after which time the filters were removed, stained, and counted as previously.<sup>12</sup> The chemotactic response was expressed as the number of cells migrating the full thickness of the filter, in ten high power microscope fields, per 10<sup>7</sup> granulocytes applied to the upper chamber. To exclude the possibility that excessively rapid passage of granulocytes through the filter, leading to their falling off into the lower chamber, and a falsely low determination of chemotactic response, the upper surface of all filters was carefully examined after cell counts had been made on the lower surface. In all instances there was a good inverse correlation between the segmented granulocytes seen on the upper and lower surfaces.

### *Activated Complement Preincubation*

In studies of granulocyte chemotactic responsiveness after incubation with activated complement, purified cobra venom was used as the activator.<sup>2,19</sup> Cobra venom-treated human AB serum was diluted to concentrations of 5.0, 2.5, 1.25, and 0.625  $\mu$ l/ml in HBSS. Washed leukocytes were divided into aliquots and suspended in each of these solutions, as well as in HBSS alone. The cell suspensions were incubated in a rocker bath at 37°C for 30 min, and then doubly washed in HBSS prior to resuspension in HBSS containing 0.5 g/100 ml HSA for the chemotaxis assay. PNH and control leukocytes were treated identically and assayed in parallel.

### Alternative Assays of Cell Viability

The viability of the cells was measured by trypan blue exclusion over a 10-min incubation at room temperature; on all occasions, greater than 96% of cells excluded the dye before and after activated complement component incubation. In addition, the ability of granulocytes to phagocytose and kill *Staphylococcus aureus* was assayed by a modification of the method of Quie<sup>12</sup> both before and after activated complement incubation.

### Lysosomal Enzyme Studies

Four lysosomal enzymes, including myeloperoxidase,<sup>21</sup> beta-glucuronidase,<sup>22</sup> lysozyme,<sup>23</sup> and alkaline phosphatase<sup>24</sup> were assayed by the referenced techniques. Lysosomal rupture was induced by sonication for 60 sec in the presence of 0.5% Triton X-100 (Calbiochem, Calif.) with careful cooling of the dextran-sedimented granulocyte-rich fraction. This method of extraction was found to produce optimal release of the four enzymes studied, and enzyme activities were expressed in terms of granulocyte numbers sonicated.

Granulocyte alkaline phosphatase was quantitated histochemically on cell fractions before and after chemotaxis.<sup>14</sup> Cells were scored either positive or negative, and to minimize observer error, the slides were read by a technician who was unaware of the type of experiment being done. Normal and PNH granulocytes were studied in this way after they had been incubated in serum previously activated with cobra venom, as above, and diluted 5.0  $\mu$ l/ml in HBSS for 30 min at 37°C. Controls were provided by incubation of both normal and PNH granulocytes in HBSS. After the cells had been doubly washed in HBSS, an aliquot for histochemical assay of the initial LAP level was taken. The cells were then placed in the Boyden chambers, and complement-mediated chemotaxis was induced by a 5.0  $\mu$ l/ml dilution of cobra venom-activated serum. After incubation, the lower surface of the Millipore filters was washed, and the cells collected were assayed for their LAP content. This score was then expressed as a percentage of the score of the total granulocyte population initially added to the chamber.

## RESULTS

### Chemotactic Responsiveness of Fresh Cells

Fresh, untreated PNH granulocytes migrated normally in Boyden chambers, regardless of the chemotaxin used (Fig. 1). All patients in Table 1, with the exception of L.M., were studied. Thus, bacterial supernatant or activated complement components, generated by either classical (immune complex-activated) or alternate (cobra venom-activated) pathways, promoted similar motility in both types of cells.

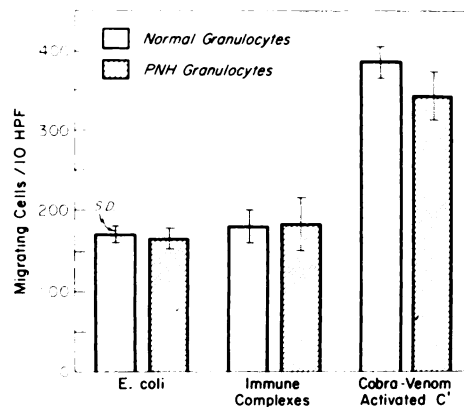
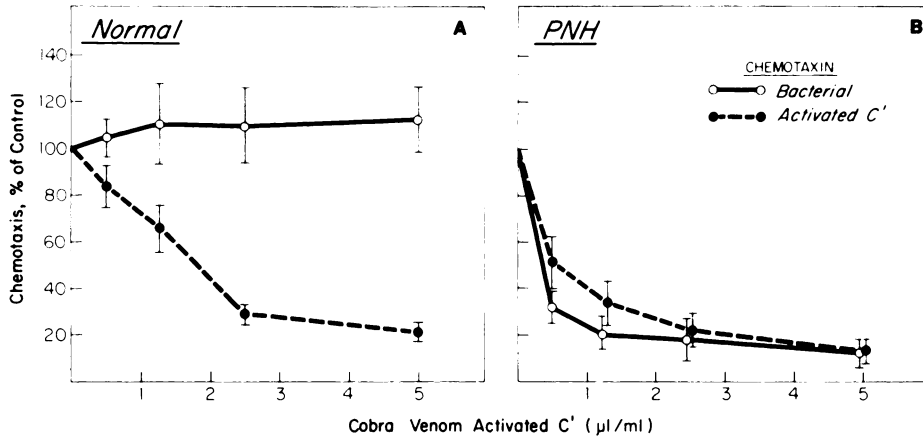


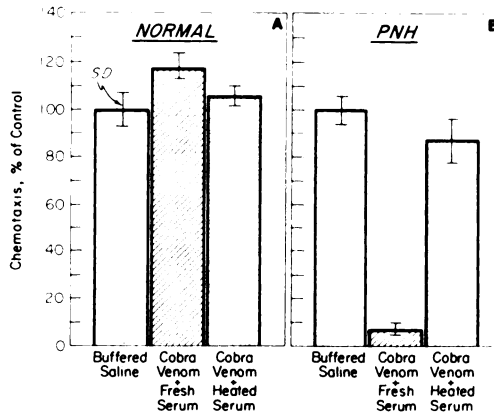
Fig. 1. Chemotactic responses of granulocytes from normals ( $n = 6$ ) and PNH patient ( $n = 5$ ) toward bacterial supernatant or complement activated by the classical (immune complexes) and by the alternate (cobra venom) pathways. No significant differences are observed.



**Fig. 2.** Effect of prior incubation with activated complement components upon chemotaxis. (A) Chemotaxis of normal granulocytes ( $n = 4$ ) is selectively depressed only toward activated complement chemotaxins (dashed lines)—motility toward bacterial supernatant (solid lines) is preserved. (B) Chemotaxis of PNH granulocytes ( $n = 4$ ) is suppressed at lower complement levels and toward both types of chemotaxins equally. Chemotactic responses at each incubation  $C'$  level is expressed  $\pm$  SE as a percentage of the response of the same granulocytes incubated in HBSS and assayed in parallel.

*Chemotactic Responsiveness Following Pre-exposure to Activated Complement Components*

In contrast, strikingly different results were noted if leukocytes were incubated with activated complement components prior to this chemotaxis assay (Fig. 2). Patients L.L., H.R., B.L., and J.O'R. were studied. At the lowest concentration of activated complement,  $0.625 \mu\text{l/ml}$ , normal granulocytes were unaffected; at higher concentrations a dose-dependent inhibition of motility occurred specifically toward the chemotaxins of activated serum complement; their motility toward bacterial chemotaxis remained normal (Fig. 2A). The motility of the PNH granulocyte, on the other hand, was markedly inhibited even after exposure to the lowest concentrations of activated complement components ( $0.625 \mu\text{l/ml}$ ) regardless of the chemotaxin utilized in the lower com-



**Fig. 3.** Obviation of complement-induced suppression of PNH granulocyte chemotaxis by heat inactivation ( $56^\circ\text{C}$ ; 30 min) of serum prior to its activation by cobra venom (B). Chemotaxis was measured toward bacterial supernatant after incubation of granulocytes in  $5 \mu\text{l/ml}$  venom-treated sera; normal granulocytes are unaffected in this system (A).

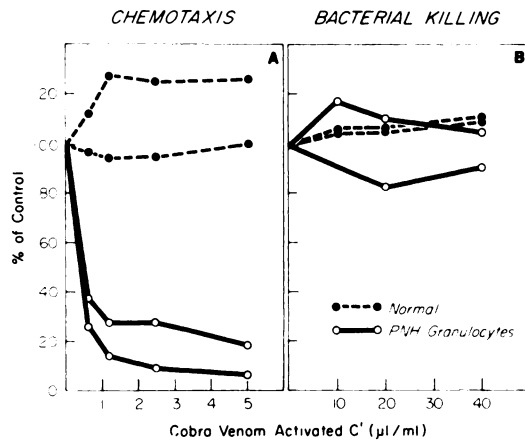


Fig. 4. Preserved phagocytic/bactericidal activity of normal (dashed lines) and PNH (solid lines) granulocytes following incubation with activated complement. At complement concentrations several-fold higher than needed to suppress PNH granulocyte chemotaxis toward bacterial supernatant (A), both normal and PNH granulocytes kill *Staphylococcus aureus* equally well (B).

partment of the Boyden chamber (Fig. 2B). Moreover, in direct contrast to normal cells, which were unaffected in their motility toward bacterial chemotaxin, PNH granulocytes developed marked inhibition of motility at the lowest concentrations of activated serum complement utilized (Fig. 2). The complement-dependent nature of this paralysis was supported by the fact that heat inactivation of the serum prior to the addition of the cobra venom prevented the inhibition (Fig. 3).

#### Alternative Viability Studies

Although incubation of PNH granulocytes with activated complement components induces these marked chemotactic defects, it produces no impairment of phagocytic/bactericidal function in these (or normal) cells (Fig. 4). This finding, in addition to the normal trypan blue exclusion manifested by all leukocytes in these studies, confirms that diminished cell viability could not underlie these results.

#### Lysosomal Enzyme Contents

As previously reported by others, we found the lysosomal enzyme content of PNH granulocytes to be virtually identical to that of normals, except for alkaline phosphatase (LAP) (Table 2). In two of the four patients studied, LAP

Table 2. Biochemical Assays of Normal and PNH Granulocyte Lysosomal Enzymes

Patient	Enzyme Activities*			
	LAP	MPO	Beta-Glucuronidase	Lysozyme
B.L.	2.34†	54†	47	40
J.O'R.	0.32†	93	55	37
L.S.	0.68†	86	76†	50†
L.M.	1.31	127	47	40
Normal range (n = 14)	1.18 ± 0.31‡	100 ± 17	45 ± 8	33 ± 6

\* The units of granulocyte enzyme activity are, respectively,  $\mu$  moles *p*-nitrophenyl phosphate/hr/ $10^7$  cells,  $\mu$ g horseradish peroxidase/ $10^7$  cells,  $\mu$ g phenolphthalein/hr/ $10^7$  cells, and  $\mu$ g eggwhite lysozyme/ $10^7$  cells.

†  $p < 0.05$  as calculated from duplicate determinations based upon the unpaired *t* test.

‡ Values are expressed as the arithmetic mean ± SD.

**Table 3. Return Toward Normal Levels of LAP in Those PNH Granulocytes Still Capable of Chemotaxis After Activated Complement Preincubation**

Granulocytes	Preincubation Medium	
	HBSS	Cobra Venom Activated Serum Diluted 5.0 $\mu$ l/ml in HBSS
Normal (n = 9)	-8 $\pm$ 6*	-19 $\pm$ 10%
PNH (n = 4)	-5 $\pm$ 5	+31 $\pm$ 10%†

\*Values represent the mean  $\pm$  SD of the percentage change in LAP scores of those granulocytes still capable of chemotaxis after incubation in either of the two media, using the LAP scores of the total cell population applied to the Boyden chamber as the 100% initial level.

†p < 0.05 as calculated in the unpaired t test.

was 2 SD or more below the normal range, while myeloperoxidase, beta-glucuronidase, and lysozyme levels were normal or actually slightly elevated.

In contrast, when PNH granulocytes from these four patients were incubated with activated complement to induce impaired chemotaxis, those few granulocytes which remained capable of migrating through Boyden chamber Millipore filters contained significantly more LAP when compared to the initial total population LAP score (Table 3). This increment was particularly striking, since, in the case of normal cells, the LAP of migrating cells was actually lower than the initial overall population by 19%.

#### DISCUSSION

This demonstration of marked intolerance of PNH granulocytes to incubation with activated serum-complement components in a dose-dependent fashion is consistent with the postulated panmyelopathic nature of the disease—a feature suspected from the frequent association of PNH with myelofibrosis and acute myelogenous leukemia.<sup>25-31</sup> Increased sensitivity to complement-mediated lysis has previously been documented for erythrocytes, leukocytes, and platelets when the cells are present during on-going complement activation.<sup>1-3,32</sup> In this study, hypersensitivity to preformed activated complement components produces surface changes on granulocytes which result in sluggish motility rather than in cellular lysis. We believe that these results are relevant to the inordinate infection susceptibility suffered by patients with PNH,<sup>6</sup> and possibly to their mild leukopenia.<sup>4,5</sup> We also suggest that the dysfunction described here is consistent with the observation that PNH granulocytes migrate poorly into inflammatory skin windows.<sup>5</sup>

It should be noted that enlargement of the dose of activated complement present during incubation to 2.5  $\mu$ l/ml (0.25% dilution of serum in HBSS) produces a selective impairment of normal granulocyte chemotaxis toward complement (Fig. 2). This dichotomy is consistent with a hypothesis that separate receptors for these two different chemotaxins exist on the granulocyte. PNH granulocytes manifest their intolerance to activated complement components by inhibition of motility of both chemotaxins, similar to normal cells incubated with much higher concentrations of activated serum complement (100–500  $\mu$ l/ml).<sup>33-35</sup> In so far as these chemotaxins probably represent the major ones operative in vivo, an infection propensity in PNH similar to that seen in other chemotactic disorders like the lazy leukocyte syndrome,<sup>8</sup> the

Chediak-Higashi syndrome,<sup>9</sup> low-complement rheumatoid arthritis,<sup>10</sup> diabetes mellitus,<sup>11</sup> and severe hypophosphatemia<sup>12</sup> should be expected.

Because the lysosomal enzyme contents (other than alkaline phosphatase) of the PNH granulocytes studied was essentially within normal limits, it is unlikely that the low alkaline phosphatase, seen in two of these patients, is related to complement-mediated lysosomal enzyme release *in vivo*. But the fact that after incubation with activated complement components those few PNH granulocytes still capable of migration in Boyden chambers possess a more normal LAP content suggests that a clonal abnormality occurs in granulocytes analogous to that described in PNH erythrocytes.<sup>1</sup> It appears that an abnormal clone of LAP-deficient leukocytes is particularly susceptible to chemotaxis inhibition by activated complement, in contrast to a more normal clone possessing normal LAP and mobility.

Our results also suggest that a vicious cycle may amplify symptomatology in PNH. Thus, an initially mild bacterial infection causing low-grade complement activation could result in acquired dysfunction of chemotaxis; this might lead to suboptimal control of the infection and to further complement activation. Since the infection accelerates hemolysis in PNH, presumably from complement activation,<sup>4</sup> we note that this proposed deleterious sequence may contribute significantly to the morbidity of this disease.

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