A Platelet and Granulocyte Membrane Defect in Paroxysmal Nocturnal Hemoglobinuria: Usefulness for the Detection of Platelet Antibodies

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ABSTRACT The tendency of platelets and leukocytes to lyse after their interaction with antibody and complement was studied by measuring the release of ^{51}Cr from cells labeled with this isotope. Platelets from six patients with paroxysmal nocturnal hemoglobinuria (PNH) were 15–230 times more sensitive to antibodies and 10–32 times more sensitive to complement than normal platelets or platelets from patients with other types of thrombocytopenic or hemolytic disorders. Mixed white blood cell (WBC) preparations from patients with PNH were 3–20 times more sensitive to anti-WBC antibodies and 5–10 times more sensitive to C′ than were WBC preparations from normal subjects, but PNH lymphocytes showed normal immunologic reactivity. PNH platelets, like PNH erythrocytes, lysed more readily than normal platelets in acidified serum and in media of reduced ionic strength, but these characteristics were not demonstrable with PNH WBC's under the conditions of study. In PNH, platelets appear to comprise a single population with respect to their sensitivity to immune lysis, yet their survival time as measured with ^{51}Cr falls within normal limits. PNH granulocytes likewise appear to consist of a single, uniformly sensitive population.

It is concluded that, in PNH, platelets and granulocytes share the membrane defect characteristic of erythrocytes in this disorder. These observations support the concept that PNH arises as the result of a somatic mutation in a primitive cell capable of differentiating into erythroblast, myeloblast, and megakaryoblast lines. PNH platelets or enzymatically treated normal platelets permit the detection of some types of platelet antibodies in dilutions up to 2000-fold greater than is possible with currently available methods, a finding suggesting that the immune lysis technique will prove useful for the study of platelet immunology.

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INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic disorder characterized by hemolytic anemia and, usually, by leukopenia and thrombocytopenia. Although rare, this disease has been the object of considerable investigation because the remarkable sensitivity of PNH erythrocytes to lysis by the complement complex makes them uniquely useful for the study of immune hemolysis and for the detection of certain types of hemolytic antibodies.

While the existence of an erythrocyte abnormality in patients with PNH seems unequivocal (2, 3), no conclusive evidence has been presented to indicate that platelets or leukocytes are qualitatively abnormal. In studies here described, the immunologic reactivity of platelets and leukocytes from patients with PNH was compared with that of normal platelets and leukocytes using release of an isotopic label, ^{51}Cr, as a measure of immune injury. The results indicate that in PNH both platelets and granulocytes share the membrane abnormality present on the erythrocytes of patients with this disease.

METHODS

10 ml of freshly drawn blood was anticoagulated with 0.3 ml of 5% ethylenediaminetetraacetate (EDTA) and centrifuged at 400 *g* for 10 min. The platelet-rich plasma (PRP) was removed and the red blood cells (RBC's) were mixed with 6 ml of isotonic NaCl and again centrifuged to remove residual platelets. The two supernatants, which contained

80–90% of the total platelets in the shed blood, were pooled and the platelets were concentrated to a volume of 0.7 ml by centrifugation at 1500 *g* for 15 min in an angle-head centrifuge. 200 μc of sodium chromate-^{51}Cr in 0.2 ml of isotonic NaCl (Iso-Serve, Division of Cambridge Nuclear Corporation, Cambridge, Mass.) was added, and the mixture was incubated for 30 min at room temperature. The labeled platelets were then washed twice with ultracentrifuged platelet-poor plasma (PPP), suspended in 1.0 ml of PPP, and counted by phase microscopy. In the final suspension approximately 98% of the total ^{51}Cr was bound to platelets, while 2% was free or bound to a small number of contaminating red cells. On the average, 1050 cpm or 0.016 μc of ^{51}Cr were bound to the 2.0×10^6 platelets used in reaction mixtures (below).

For determination of sensitivity to *antibodies*, labeled platelets were diluted to a concentration of 20,000/mm^3 in fresh, ABO-compatible PPP which served as the source of complement (C′) in the reaction mixtures. 0.1-ml aliquots of the labeled platelet suspension were added to tubes containing 0.05 ml of antibody (heated at 56°C for 30 min) and 0.025 ml of 0.05 M magnesium chloride. The magnesium was sufficient to saturate the excess EDTA present in the PRP and produce a final magnesium ion concentration of approximately 0.005 moles/liter. Lysis curves were unaffected by a twofold decrease or increase in this concentration of magnesium. For studies with quinidine or quinine-dependent antibodies, 0.025 ml of either quinine sulphate or quinidine gluconate, 0.002 M, were added to each of the tubes with a dropping pipette (Cooke Engineering Company, Alexandria, Va.). The reaction tubes were incubated for 2 hr at 37°C. 2.0 ml of isotonic NaCl containing 0.5% EDTA was then added, and the tubes were centrifuged for 30 min at $600 \times g$ in an angle-head serological centrifuge (Serofuge, Clay-Adams, Inc., New York). The supernatants, containing the ^{51}Cr released from platelets, were transferred to counting tubes, and their content of ^{51}Cr was determined in a well-type scintillation counter (Gamma-Guard, Tracerlab Div., Laboratory for Electronics Inc., Waltham, Mass.). Control studies showed that with drug-dependent antibodies immune lysis of platelets required the presence of both drug and antibody and did not occur in the absence of either. Under the conditions described, the small amount of calcium and magnesium added with antibody did not affect lysis curves, and neither gross nor microscopic clotting occurred in the reaction mixtures. Fresh EDTA plasma and serum were equally effective as sources of C′ for antibody titrations provided that magnesium was added in the case of plasma. For most studies plasma was used because platelets could be resuspended more readily, and lysis curves were more reproducible in this medium. Plasma or serum from approximately 20% of normal donors was unsuitable because excessive (> 25%) "spontaneous" lysis of PNH platelets occurred in control tubes containing plasma and magnesium but no antibody or drug. This phenomenon could not be related to any previous immunization, and its cause is presently undetermined.

For the determination of their *complement* sensitivity, labeled platelets were washed twice with heated EDTA PPP and suspended in isotonic NaCl at a concentration of 40,000/mm^3. 0.05 ml of this suspension was added to tubes containing 0.2 ml of fresh EDTA PPP serially diluted in PPP which had been heated at 56°C for 60 min. 0.025 ml of 0.1 M magnesium chloride, 0.05 ml of quinine-dependent antibody diluted 1:3 in isotonic NaCl, and 0.025 ml of 0.002 M quinine sulfate were then added. The mixtures were incubated for 2 hr at 37°C, and the per cent of ^{51}Cr released was determined as described above for antibody titrations. Similar results were obtained when fresh serum diluted in heated serum was used as the source of C′ rather than plasma. Heated plasma rather than buffer was used as the diluent for C′ because excessive "spontaneous" lysis occurred when platelets were suspended in a low-protein medium at 37°C. By use of this system of C′ titration, lysis curves of sheep red cells coated with rabbit amboceptor were essentially the same as when C′ was diluted in buffer in the conventional manner.

In preliminary studies, difficulty was encountered in establishing a constant reference source of C′, as it was found that antibody and C′ titers varied when plasma samples from different normal donors were used as the source of C′. Moreover, frozen plasma rapidly decreased in potency as a C′ source even when kept at −80°C. We were unable, therefore, to maintain a stable source of C′ for comparative studies of platelet lysis. Regardless of the C′ source, however, the sensitivity to antibody or C′ of a particular PNH platelet preparation *relative* to normal platelets was the same. Therefore, in comparing the immunologic sensitivity of PNH and normal platelets, the *ratio* of the quantity of antibody or C′ required to lyse 50% of normal platelets to the amount required for comparable lysis of PNH platelets was used as the measure of sensitivity, rather than the absolute titer of antibody or C′.

Under the conditions of labeling, 80 ±1.3% of platelet-^{51}Cr could be released in a soluble form when platelets lyse,[1] but the remaining 20% was bound to membraneous components of the cell and was sedimented by centrifugation despite total lysis. Accordingly, the percentage of total ^{51}Cr released was divided by 0.8 to determine the actual percentage of cells lysed. In studies with normal platelets, 4–8% of ^{51}Cr was released in control tubes containing no antibody. With PNH platelets "spontaneous" release of isotope ranged from 5 to 25%. When antibody was present in the reaction mixtures, total lysis was the consequence of both "spontaneous" and immunologic lysis. The contribution of immunologic lysis could not be determined simply by subtracting from total lysis the amount of "spontaneous" lysis which occurred in control tubes because with greater amounts of immunologic lysis a lesser degree of "spontaneous" lysis would be expected. An equation was derived, therefore, to calculate y, the fraction of total platelets lysed immunologically in each reaction tube, as given in Appendix I. In some studies the log of $y/1 - y$ was plotted against the log of complement or antibody concentration as done by Rosse and Dacie in analyzing the lysis curves of PNH erythrocytes (6). This transformation (7) converts the sigmoid curves of immune lysis to curves which approximate straight lines throughout most of their extent and simplifies the analysis of mixed cell populations which vary in their sensitivity to antibody or C′.

Papain treatment of normal platelets. An aliquot of ^{51}Cr-labeled platelets was washed twice with ABO-compatible, EDTA PPP which had been heated at 56°C for 30 min and centrifuged to remove precipitated fibrinogen. The washed platelets were suspended in phosphate-buffered (pH 7.4) isotonic NaCl to a final concentration of 2×10^6/mm^3. 0.9 ml of buffer containing 0.3 mg crystalline papain (Sigma

[1] Very little information is available regarding the nature of the platelet-chromate bond (4, 5). Our own preliminary studies show, in accord with those of Davey (5), that 70% of ^{51}Cr in platelet lysates is TCA-soluble and can be dialyzed (Aster, R. H. Unpublished observations), indicating that most of the label is bound to a relatively small molecule.

Chemical Co., St. Louis, Mo.) and 0.6 mg crystalline L-cysteine (Sigma) per ml was warmed to 37°C. 0.1 ml of the platelet suspension was added, and the mixture was incubated at 37° for 15 min. The platelets were then washed twice with heated EDTA PPP, suspended in 1 ml of PPP, and recounted for use in experimental studies. Papain-treated platelets underwent excessive (> 25%) spontaneous lysis in plasma from 75% of normal persons. Plasma from the remaining 25% of normal donors was used as the source of C′ in studies with the enzymatically treated cells.

^{51}Cr labeling and lysis of mixed WBC and lymphocyte preparations. Immune lysis of leukocytes was studied by a modification of the methods of Sanderson (8) and Rogentine (9). 10 ml of whole blood was anticoagulated with 1.5 ml of acid citrate dextrose (ACD) solution (National Institutes of Health [NIH] formula A). 2 ml of 6% dextran in isotonic NaCl (Intradex, Glaxo Laboratories, Ltd., Greenford, England) was added, and the erythrocytes were allowed to settle for 30 min at 37°C. The supernatant, containing white blood cells (WBC's) and platelets, was centrifuged at 150 *g* for 10 min. The resulting WBC button was suspended in 3 ml of EDTA PPP and centrifuged for 30 sec in an angle-head centrifuge (Serofuge, Clay-Adams, Inc., New York). The WBC button was suspended in 0.7 ml of EDTA PPP and incubated at room temperature with 200 μc of sodium chromate-^{51}Cr. The WBC's were then washed twice with cold EDTA PPP by centrifuging for 30 sec in the Serofuge. The WBC button was then resuspended in 2 ml of 1% ammonium oxalate to lyse residual RBC's. The WBC's were washed twice more with cold EDTA PPP and were suspended in 1 ml of EDTA PPP and kept at 5°C until used. With WBC, approximately 85% of the radioactive label was released in a soluble form when cells were lysed osmotically in distilled water. Differential counts on the WBC preparation were essentially the same as in the original whole blood. The ratio of WBC's to platelets in the final preparation was approximately 10:1.

For determination of *antibody* sensitivity 0.05 ml of WBC diluted to 5000/mm³ in isotonic saline and containing about 0.015 μc of ^{51}Cr were added to tubes containing 0.1 ml of fresh, ABO-compatible, EDTA plasma, 0.025 ml of 0.05 M magnesium chloride, and 0.05 ml of serially diluted, heated, anti-WBC antibody prepared in rabbits immunized with semipurified preparations of human WBC's in incomplete Freund's adjuvant. The mixtures were incubated for 60 min at 37°C. 1.5 ml of EDTA-isotonic saline was then added, the tubes were centrifuged for 5 min in the Serofuge, and the supernatants were decanted for determination of released radioactivity. Studies of the *complement* sensitivity of labeled WBC's were essentially as described for platelets except that 0.05 ml of heated rabbit anti-WBC antibody diluted 1:3 in isotonic saline, rather than platelet antibody, was added to each tube.

For preparation of lymphocytes, 12 ml of blood was defibrinated with glass beads. The residual blood was sedimented with dextran, and the supernatant was concentrated by centrifugation at 150 *g* to a 2 ml volume in the serum-dextran supernatant. Granulocytes and monocytes were removed by incubating the WBC on a glass bead column essentially as described by Rabinowitz (10). The lymphocytes in the column effluent were concentrated, labeled with ^{51}Cr, and washed free of RBC as described for whole WBC preparations. The final preparations contained 95–100% lymphocytes, and the ratio of lymphocytes to platelets ranged from 10:1 to 100:1. ^{51}Cr content per cell was two-thirds that of mixed WBC preparations. Titration of antibody and complement with labeled lymphocytes was performed as described for mixed WBC preparations.

In each study in which labeled WBC or lymphocytes were used, control tubes were included in which the labeled leukocytes were incubated with 0.05 ml of platelet antibody having anti-$P1^{A1}$ specificity (11) which was sufficiently potent to lyse any contaminating platelets but did not react with WBC. It was found that platelet radioactivity never con-

TABLE I

Clinical and Hematologic Data in Patients with PNH

			Hematologic values					Clinical			
Patient	Sex	Age	Hct	Reticulocytes	WBC	Platelets	Platelet survival time*	Presenting state	Duration of illness	Splenectomy	Present state
		yr	%	%	*per mm³*	*per mm³*	*days*				
J.D.	M	19	40	2.0	6100	214,000	9.5	Hemolytic anemia	2 yr	No	Intermittent hemolysis with infections
R.D.	M	28	35	4.0	4700	107,000	—	Aplastic anemia	3 yr	No	Occasional hemolysis
J.M.	F	33	36	2.4	3900	120,000	8.5	Hemolytic anemia	14 yr	No	Intermittent hemolysis with infections
M.P.	M	63	22	0.3	7200	220,000	9.0	Hemolytic anemia	20 yr	Yes	Severe renal failure due to hemosiderosis
K.B.	F	67	29	7.0	4000	100,000	—	Hemolytic anemia	2 yr	No	Persistant hemolysis
E.O.	M	72	48	1.3	9500	44,000	—	Aplastic anemia	1 yr	No	Anemia responded to androgen therapy

PNH, paroxysmal nocturnal hemoglobinuria; Hct, hemocrit, WBC, white blood cell.

* Normal value 8–10 days.

stituted more than 2% of total cell-bound radioactivity of the leukocyte preparations and thus did not significantly influence the lysis curves.

Acid lysis of platelets, leukocytes, and erythrocytes. Fresh ABO-compatible normal serum was adjusted to pH values ranging from 5.5 to 8.0 by addition of 0.2 N HCl or 0.2 N NaOH, and 0.4 ml of each serum sample was transferred to a 10 × 75 mm glass tube. 0.05 ml of washed, ^{51}Cr-labeled platelets at concentration 100,000/mm^3 in isotonic NaCl were then added, and the mixture was immediately covered with several drops of mineral oil to prevent any shift in pH due to loss of CO_2. For lysis of labeled WBC, lymphocytes, or RBC, the procedure was similar except that 0.05 ml of WBC or lymphocytes at concentrations of 8000/mm^3 or 0.05 ml of a 25% suspension of washed RBC was added. The mixtures were incubated at 37° for 60 min. 1.5 ml of EDTA-saline was then added, the tubes were centrifuged, and their supernatants were decanted for determination of released radioactivity.

"Sucrose" lysis of platelets, leukocytes, and erythrocytes. 0.05 ml of washed platelets at concentration 100,000/mm^3 or 0.05 ml of washed, labeled WBC's at concentration 5000/mm^3 in isotonic NaCl were added to 0.2 ml of fresh EDTA PPP. 0.025 ml of 0.1 M magnesium chloride and 1.8 ml of either 10% sucrose solution or isotonic NaCl solution were added to each tube. The mixtures were incubated for 30 min at 37°C. 1.5 ml of EDTA–isotonic NaCl was then added, the tubes were centrifuged and the supernatants decanted for determination of released radioactivity. Sucrose lysis of RBC's was carried out as described by Jenkins, Hartmann, and Kerns (12).

Assays for drug-dependent platelet antibodies by complement fixation (13, 14), platelet agglutination (15), inhibition of clot retraction (15) and platelet factor 3 release (16) were as described. Platelet survival times were determined by labeling autologous platelets with ^{51}Cr (17).

Selection of patients. Criteria for the diagnosis of PNH in the patients whose platelets were used in the present study were: acquired hemolytic disease with negative anti-

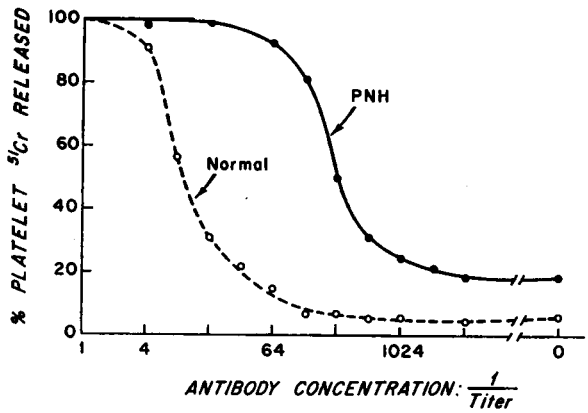

FIGURE 1 Lysis of paroxysmal nocturnal hemoglobinuria (PNH) and normal platelets by serial dilutions of quinine-dependent platelet antibody. PNH platelets lysed with approximately one-thirtieth the amount of antibody required for comparable lysis of normal platelets.

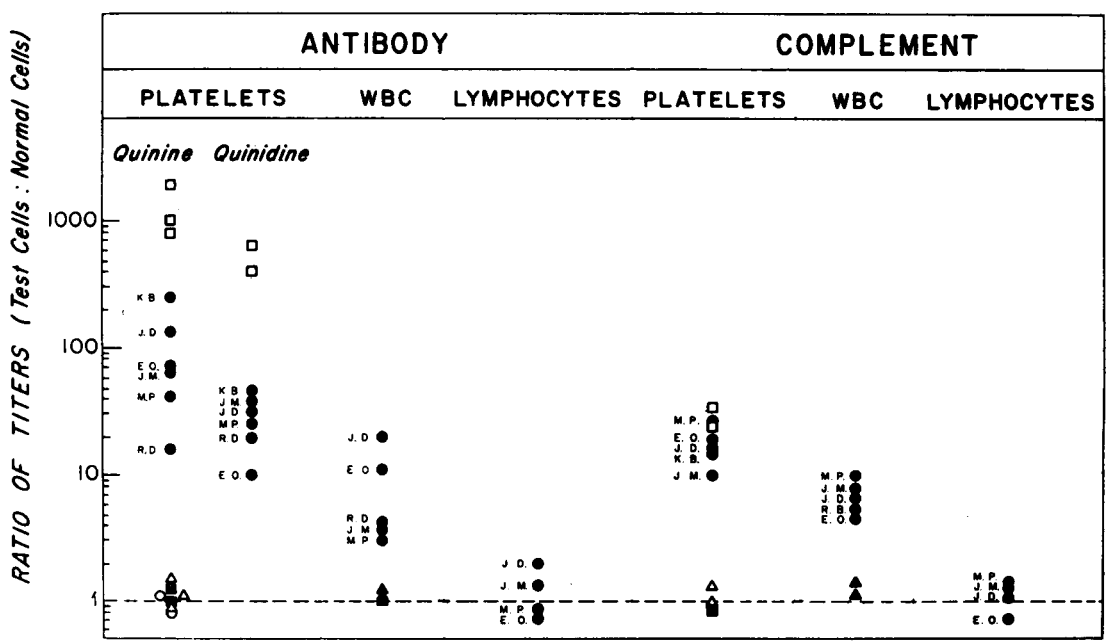

FIGURE 2 Lysis of platelets, whole white blood cell (WBC) preparations, and lymphocytes from patients with PNH and other disorders by serial dilutions of antibodies and complement. Ordinate gives the ratio of the inverse titer of antibody or C′ which caused 50% lysis of test platelets, WBC, or lymphocytes to that required for 50% lysis of normal cells tested simultaneously. ● = PNH, △ = idiopathic thrombocytopenic purpura (ITP), ■ = "autoimmune" hemolytic anemia, ○ = "hypersplenic" thrombocytopenia, ▲ = "hypersplenic" leukopenia, □ = papain-treated normal platelets.

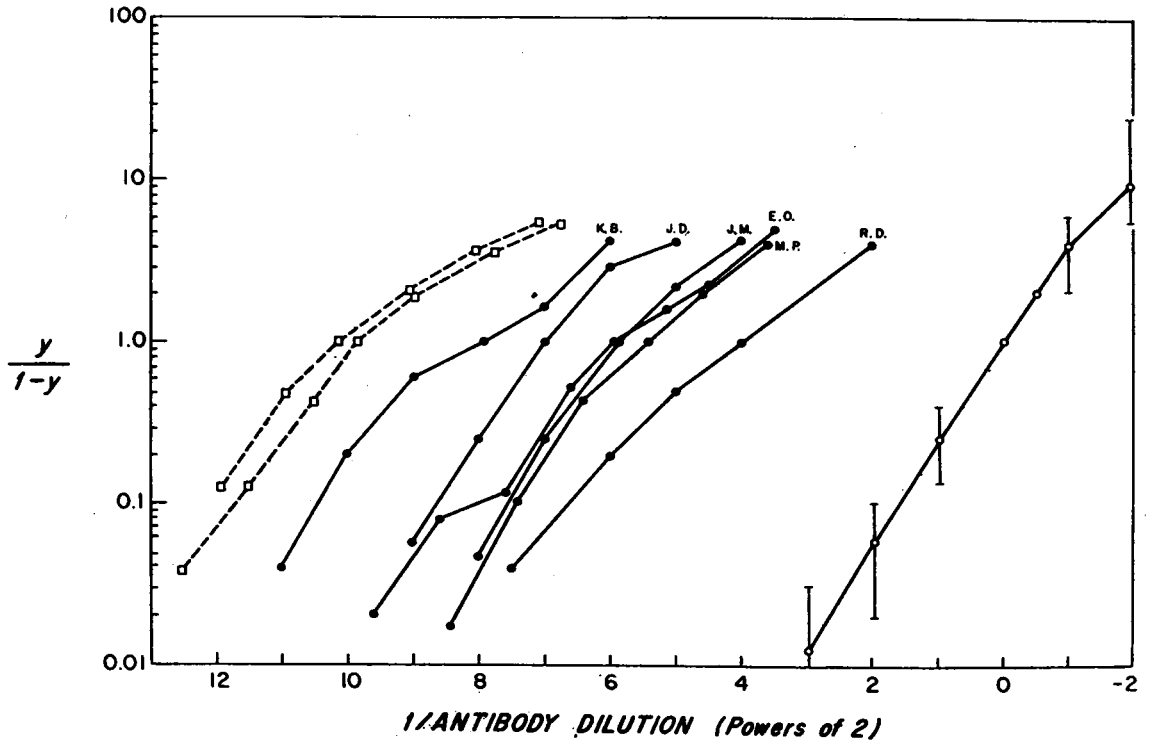

FIGURE 3 Lysis of normal platelets (○—○), PNH platelets (●—●), and papain-treated normal platelets (□- - -□) by serial dilutions of quinine antibody using the logarithmic transformation of the Von Krogh equation. y = fraction of total platelets lysed immunologically. Value of 1.0 on the ordinate represents 50% immune lysis. In each study, the amount of antibody causing 50% lysis of normal platelets was arbitrarily taken as 1.0 (value of zero on the abcissa), and the lysis curves obtained with PNH or papain-treated platelets studied simultaneously were plotted relative to this standard as described in Methods. The normal curve gives the average values obtained in eight studies with normal platelets ± 2 SEM.

globulin (Coombs) and positive acid hemolysis and sucrose lysis tests on erythrocytes. Hemosiderinuria was present in each of the patients studied. Clinical and hematologic data obtained at the time studies were carried out are summarized in Table I.

RESULTS

Antibody sensitivity. Platelets from each of the six patients with PNH were incubated with each of two platelet antibodies. One antibody, obtained from a patient with quinine-induced thrombocytopenic purpura, fixed complement in a dilution of 1:8, agglutinated platelets in a dilution of 1:4, inhibited clot retraction in a dilution of 1:2, and caused release of platelet factor 3 in dilution 1:4. The second antibody from a patient with quinidine sensitivity fixed C′ in dilution 1:32, agglutinated platelets in dilution 1:2, inhibited clot retracton in dilution 1:4, and released platelet factor 3 in dilution 1:8. These antibodies were selected because within the limits of experimental error they reacted similarly with platelets of each of seven normal subjects tested, in con-

TABLE II

Sensitivity of PNH Platelets (Patient M.P.) and Normal Platelets to Eight Platelet Antibodies

Antibody specificity	Titer*		Ratio of titers PNH: Normal
	PNH	Normal	
Quinine	400	6	67
Quinine	240	8	30
Quinine	14	2	7
Quinidine	750	40	19
Quinidine	80	4	20
Quinidine	8	2	4
P^{A1}	500	16	31
P^{A1}	400	12	33

PNH, paroxysmal nocturnal hemoglobinuria.
* Inverse of antibody dilution which caused 50% immunologic lysis.

trast to isoantibodies reacting with specific platelet antigens which may vary widely in reactivity with platelets from different donors (11, 18). Fig. 1 shows the uncorrected lysis curves which resulted when quinine antibody was incubated in serial dilution with normal platelets and platelets from patient M. P. For release of 50% of total ^{51}Cr, about 30 times as much antibody was required for normal platelets as for the PNH platelets. In Fig. 2 is summarized the results of incubating quinidine and quinine-dependent antibodies with PNH platelets, papain-treated normal platelets, and platelets from patients with other thrombocytopenic and hemolytic disorders. PNH platelets were 15–230 times as sensitive to quinine antibody and 10–45 times as sensitive to quinidine antibody as were normal platelets. Platelets from patients with idiopathic thrombocytopenia, "hypersplenic" thrombocytopenia, and "autoimmune" hemolytic anemia were normally sensitive to antibody, but normal platelets treated with papain lysed with approximately 1/1000th the quantity of antibody required for normal platelets. In Fig. 3 is shown the Von Krogh transformations of the lysis curves obtained when quinine antibody was incubated in serial dilution with platelets from each of the six patients with PNH. On the ordinate, the values of 10, 1, and 0.1 correspond to 91, 50, and 9% immunologic lysis, respectively. The resulting lysis curves were relatively continuous throughout their extent with no apparent points of inflection as would be expected if PNH platelets consisted of two or more populations which varied in their antibody sensitivity.

The sensitivity of platelets from PNH patient M.P. to six drug-dependent platelet antibodies and two isoantibodies with $P1^{A1}$ specificity[2] (11) is compared with that of normal platelets in Table II. PNH platelets were 4–67 times more sensitive to antibody than were normal platelets with the various antibodies used.

Complement sensitivity of PNH platelets. When quinine antibody concentration was held constant and C′ concentration was diminished by serial dilution it was found that PNH platelets were 10- to 30-fold more sensitive to C′ than were normal platelets. Papain-treated platelets also showed approximately a 30-fold increase in C′ sensitivity, but platelets from three patients with other thrombocytopenic and hemolytic disorders were normally sensitive (Fig. 2). In Fig. 4 it may be seen

[2] One of these antibodies was kindly supplied by Dr. Manuel Kaplan of Washington University School of Medicine, St. Louis, Mo.

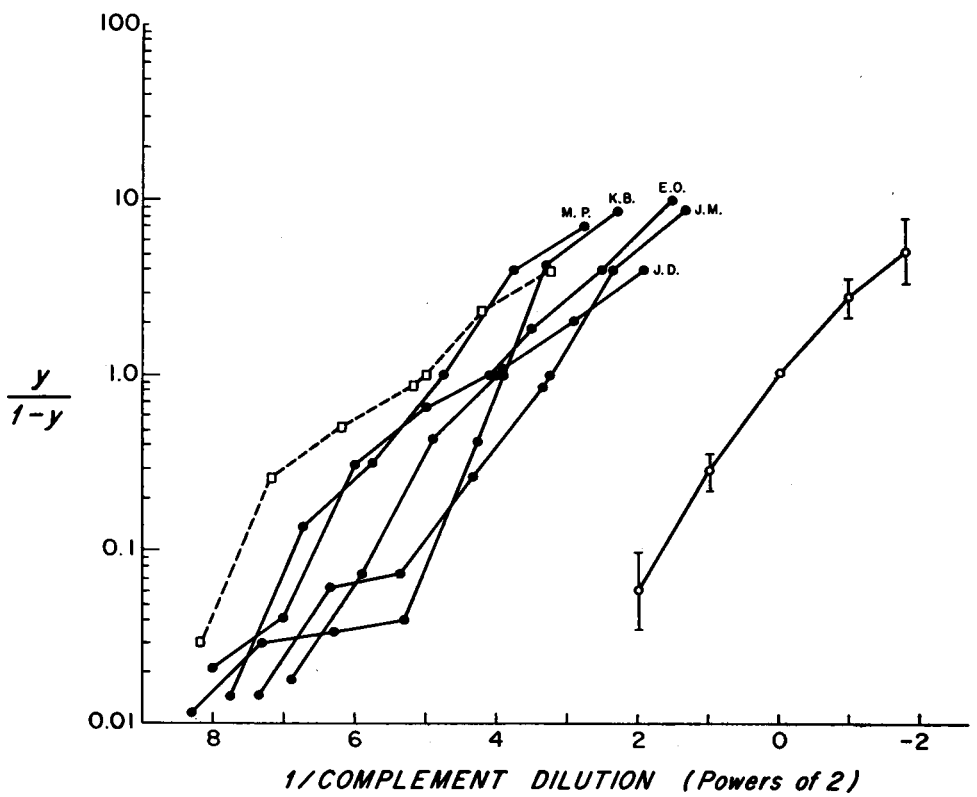

FIGURE 4 Lysis of normal platelets (○—○), PNH platelets ●—●), and papain-treated normal platelets (□- - -□) by quinine antibody with complement serially diluted. Data plotted as in Fig. 3.

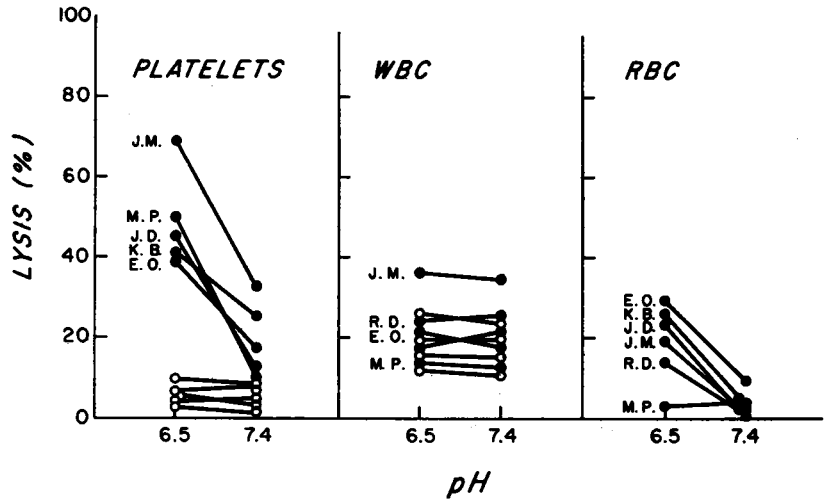

FIGURE 5 Lysis of PNH (●—●) and normal (○—○) platelets, white blood cells, (WBC's) and red blood cells (RBC's) in fresh normal serum at pH 6.5 and 7.4. Conditions as described in Methods. With normal RBC's (not shown), no significant lysis occurred at either pH.

that the lysis curves resulting when C′ was serially diminished in the reaction mixtures are also relatively continuous. Only with patients K.B. and J. M. was there any suggestion of an inflection point indicative of two platelet populations varying in sensitivity to C′.

Mixed WBC's prepared from five of the patients with PNH were also abnormally sensitive to both antibodies and complement as shown in Fig. 2, although the contrast with normal cells was not as great as with platelets. The WBC preparations tested were, nonetheless, 3- to 20-fold more sensitive to antibody and 5- to 10-fold more sensitive to complement than were normal WBC's. In contrast to whole WBC preparations, lymphocytes obtained from four of the patients with PNH did not differ significantly from normal in their sensitivity to either antibody or C′ (Fig. 2).

Acid and sucrose lysis of PNH platelets, WBC's, and leukocytes. 38–68% of PNH platelets lysed when incubated in normal serum at pH 6.5 under oil, while only 10–24% were lysed at pH 7.4 (Fig. 5). When heated serum was used or when sufficient EDTA was added to bind free calcium and magnesium, less than 10% of the

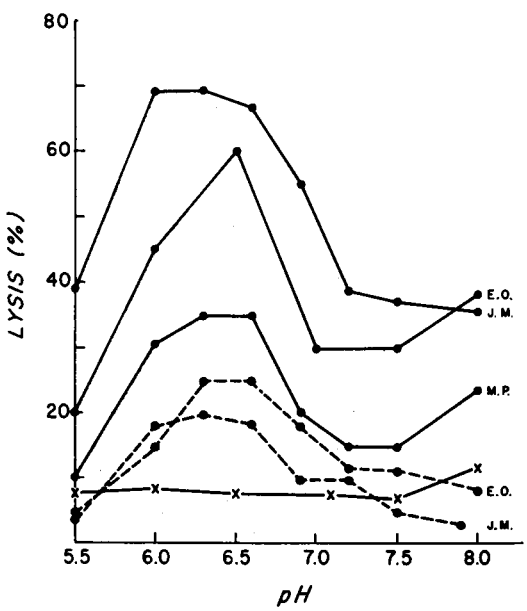

FIGURE 6 Lysis of PNH platelets (●—●), PNH RBC's (●- -●) and normal platelets (×—×) in fresh serum at varying pH. The optimum pH for lysis of both platelets and erythrocytes was approximately 6.4.

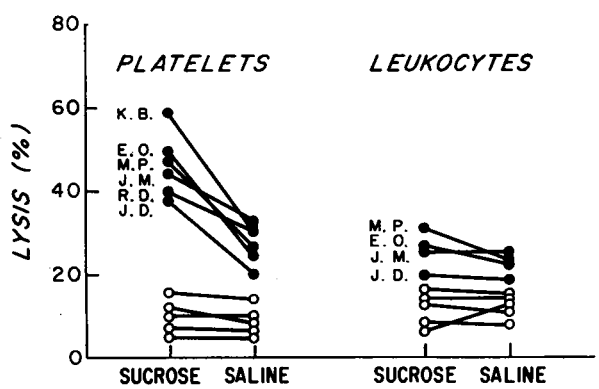

FIGURE 7 Lysis of PNH (●—●) and normal (○—○) platelets and WBC's after addition of sucrose or isotonic NaCl to magnesium-enriched ethylenediaminetetraacetate (EDTA) plasma containing labeled cells.

PNH platelets lysed at either pH. The same alteration in pH did not affect the release of ^{51}Cr from normal platelets. The optimum value of pH for lysis of platelets from three PNH patients studied (Fig. 6) was approximately 6.4. With mixed WBC and lymphocyte preparations, release of ^{51}Cr was not affected by the pH of the incubation medium under the conditions of study. Erythrocytes from five of the six PNH patients released from 15 to 40% of their hemoglobin at pH 6.5, considerably less than the percentage of platelet lysis observed under comparable experimental conditions. The optimum for red cell lysis was also 6.4. RBC's from patient M.P. did not lyse at acid pH, probably because of severe renal failure present at the time of study which greatly reduced endogenous RBC production. Consequently, nearly all his circulating erythrocytes had been transfused. Before the onset of renal failure M.P. had a documented positive RBC acid lysis test.

When the ionic strength of the reaction mixture was reduced by adding 10% sucrose, more ^{51}Cr was released by PNH platelets than when an equal amount of isotonic NaCl was added (Fig. 7). This difference was abolished when plasma was heated or when magnesium was not added. "Sucrose lysis" of platelets could not be demonstrated when citrated plasma or serum were used as the source of C′, although PNH erythrocytes did lyse under the latter conditions. With mixed WBC preparations, release of ^{51}Cr was the same when either sucrose or isotonic NaCl were added.

Antibody absorption and quantitative C′ fixation by PNH platelets. PNH platelets absorbed the same amount of quinine antibody as did normal platelets (Fig. 8). Quantitative C′ fixation studies with the same antibody showed that PNH and normal platelets were equally efficient in terms of the amount of complement fixed per platelet (Fig. 9).

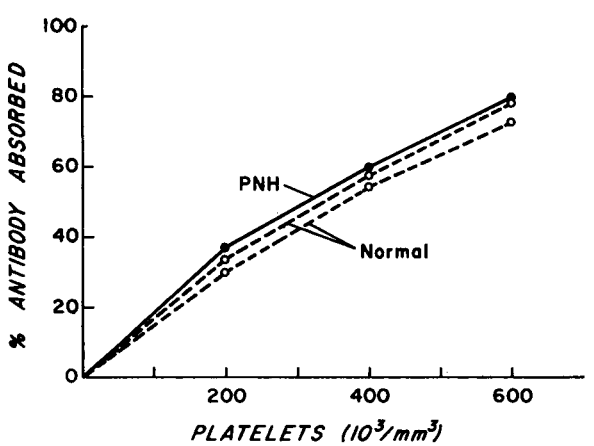

FIGURE 8 Absorption of quinine antibody containing 10^{-4} M quinine sulfate by PNH (●—●) and normal (○—○) platelets at various concentrations. After incubation for 60 min at 37°C, the mixtures were centrifuged and the supernatants were titered for residual antibody by quantitative lysis of normal platelets.

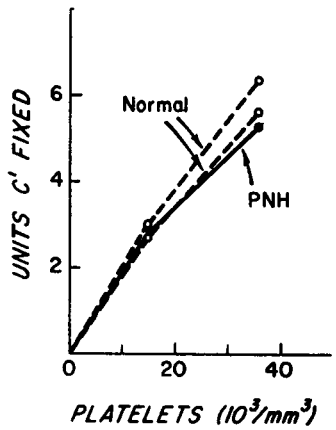

FIGURE 9 Quantitative complement fixation by PNH (●—●) and normal (○—○) platelets using quinine antibody. Reaction mixtures contained 0.05 ml of antibody, 0.05 ml of 10^{-3} M quinine sulfate, 12 50% hemolytic units of guinea pig C′, and platelets at the concentrations shown. Final volume of the reaction mixtures was 0.4 ml.

Survival of ^{51}Cr-labeled platelets. The survival times of ^{51}Cr-labeled platelets were determined in three patients. The percentage of transfused platelets which were recovered from the circulation 2 hr post-injection fell within normal limits (58–80%), and the survival times were within the normal range (Table I). Kinetics of platelet disappearance gave no evidence for the existence of two platelet populations differing in their rates of removal from the circulation.

Correlation of clinical data with the degree of leukocyte and platelet abnormality. Analysis of the clinical data given in Table I and Fig. 2 showed that in our patient population there was no significant correlation between the degree of thrombocytopenia, leukopenia, or hemolysis in a given individual and the sensitivity of platelets or leukocytes from the same patient to antibodies or C′.

DISCUSSION

Many studies have indicated that the tendencies of PNH erythrocytes to lyse in media of low ionic strength, in acidified serum, and in the presence of highly dilute antibodies and C′ are all due to increased sensitivity to the complement complex (19, 20), but the precise nature of the membrane defect which is presumed to impart C′ sensitivity to PNH red cells remains unknown. Although thrombocytopenia and granulocytopenia occur frequently in PNH, evidence that these blood elements are qualitatively abnormal has been equivocal. With respect to platelets, both Crosby (21) and

Nussey and Dawson (22) suggested that PNH platelets are abnormally fragile upon prolonged incubation, but Flexner, Auditore, and Hartmann found that PNH platelets functioned normally in coagulation tests (23), and other workers found that the concentration of PNH platelets did not diminish (19), and their capacity for clot retraction was unimpaired (24) after incubation in acidified serum. In the latter studies, it may be that observation by ordinary light microscopy did not detect minor degrees of platelet injury or that insufficient numbers of platelets were lysed to affect clot retraction, which requires rather few platelets when plasma is recalcified. Crosby also suggested that PNH granulocytes were abnormally fragile in vitro (21) and Vajda, Tóth, and Fonyodi demonstrated that their phagocytic capacity was diminished (25), an observation later confirmed by Frame, Rebuck, and Riddle (26) and Penny and Galton (27). Beck and Valentine found diminished leukocyte alkaline phosphatase (LAP) in granulocytes of patients with PNH (28), and Lewis and Dacie correlated the decrease in LAP with the severity of hemolysis (29). It was also shown by Ross and Rosenbaum that in one patient with PNH, leukocyte acetylcholinesterase was diminished (30), as is true of PNH red cells. Despite this suggestive evidence, Dacie, in a recent comprehensive summary of the curent knowledge of PNH, regards the question of a qualitative defect in cells other than the erythrocyte as "still unsettled." (31).

The present study appears to be the first in which the sensitivity to antibodies and complement of platelets and leukocytes from patients with PNH has been compared with that of cells from normal subjects. It is evident from Figs. 1–5 that, as measured by the release of ^{51}Cr, PNH platelets share the characteristics of the "sensitive" fraction of the PNH erythrocyte population in undergoing immune lysis far more readily than normal platelets as judged by either antibody (Fig. 3) or C′ (Fig. 4) sensitivity. "Spontaneous" lysis of PNH platelets in both acidified serum and in media of low ionic strength was also greater than normal and even greater than the degree of lysis which erythrocytes from the same patients underwent under comparable conditions (Figs. 5–7). The optimum pH for acid lysis of both platelets and erythrocytes from our patients was approximately 6.4 (Fig. 6), a value lower than that of about 6.8–7.0 obtained by other workers (19, 32, 33), but close to that of 6.5 described by Yachnin and Ruthenberg (34) and Kan and Gardner (35) and identical with the value of 6.4 reported recently by Kamn, Mengel, Meriwether, and Ebbert (36). No explanation has been advanced for the apparently discrepant values obtained by various workers for the pH optimum, but it may be significant that the lower values of pH (6.4–6.5) have been obtained when the reaction mixtures were overlain with oil to prevent a change in pH during incubation. WBC preparations from the five PNH patients studied also showed significantly increased sensitivity to both antibody and C′ (Fig. 2), although no abnormality in PNH WBC's could be detected with acidified serum or by lowering the ionic strength of the incubation medium. One other characteristic of PNH erythrocytes is diminished acetylcholinesterase activity (37). We were unable to detect this activity even on normal human platelets using a colorimetric technique (38) in confirmation of Zajicek (39).

Together, these observations strongly suggest that platelets, and very probably granulocytes as well, share the membrane abnormality present in erythrocytes of patients with PNH. Normal absorption of antibody by PNH platelets (Fig. 8) and their normal behavior in quantitative C′ fixation studies (Fig. 9) are comparable to observations made with PNH erythrocytes (6) and indicate that with platelets, as with red cells, there is no peculiarity of cell-antibody interaction, but, rather, that a membrane abnormality greatly enhances the lytic efficiency of a given amount of C′ activated at the cell surface.

In several important respects platelets and granulocytes of patients with PNH appear to differ from erythrocytes. Firstly, platelets sampled from the general circulation of the six patients studied appeared to belong to one platelet population which was 10–30 times more sensitive to C′ and 15- to 230-fold more sensitive to quinine antibody than were normal platelets. Possible exceptions are patients K.B. and J.M. in whom 4 and 6% of platelets respectively appeared to be slightly more sensitive than the remaining platelet population. PNH erythrocytes, on the other hand, nearly always consist of two populations: one highly sensitive to immune lysis and the other only slightly more sensitive than normal (6). In the case of nonfractionated WBC preparations, lysis curves (not shown) suggested the presence of a "sensitive" population containing 80–90% of total ^{51}Cr and an "insensitive" population containing 10–20%. Since the lymphocytes in these preparations contained 10–20% of total ^{51}Cr, these observations are consistent with the possibility that in PNH granulocytes, like platelets, constitute a single population abnormally sensitive to C′ and antibody.

A second difference between PNH platelets and erythrocytes is that, despite being highly sensitive to both antibodies and C′, PNH platelets have a normal life-span (Table I), a finding confirming the earlier observations of Cohen, Gardner, and Barnett (40) and Hartmann and Jenkins (24). This finding is particularly surprising in view of the exquisite sensitivity manifested by platelets in vivo to a variety of antibodies (11, 41, 42). We have found that serum and plasma taken from two of the

patients with PNH were as effective as normal plasma as a source of C′ for platelet lysis, a finding suggesting that failure of PNH platelets to lyse in vivo is not due to deficiency of a plasma factor required for lysis. We have also been unable to show with certainty in vitro that a mass of RBC's equal to that which surrounds platelets in vivo exerts a "protective" effect which might spare platelets from lysis (Aster, R. H. Unpublished observations). It has generally been assumed that the same membrane abnormality which sensitizes the PNH erythrocyte to in vitro lysis is also responsible for hemolysis in vivo, although there has been at least one observation to the contrary (43). The normal survival of PNH platelets suggests that a reevaluation of this concept may be in order, and that some as yet unrecognized mechanism may cause destruction of PNH erythrocytes in vivo.

Several other implications of the present study seem deserving of comment. Auditore, Hartmann, Flexner, and Balchum (44), and subsequently others (3, 45, 46), have suggested that PNH originates as a somatic mutation in the marrow, permitting some patients to "escape" from a state of marrow hypoplasia. Our studies indicate that if PNH results from such a mutation, it must occur in a primitive cell capable of differentiating into erythroblast, megakaryoblast, and myeloblast lines, in which case our data provide support for the existence of a "stem cell" in human marrow. On the other hand, the origin of the platelet membrane is rather different than that of erythrocytes or granulocytes (47). Moreover, a variety of chemical procedures are known to bestow many of the properties of PNH erythrocytes upon normal RBC's (36, 48, 49). The latter facts suggest the unlikely but conceivable possibility that the PNH abnormality is acquired by maturing cells from some extracellular influence present in the marrow cavity rather than from a genetic transformation.

Our results also suggest that quantitative platelet lysis may prove useful in the study of platelet immunology. With the six drug antibodies and two isoantibodies studied thus far, release of ^{51}Cr from normal platelets was at least as sensitive an indicator for antibody as was quantitative C′ fixation and was superior in this respect to agglutination, inhibition of clot retraction, and release of platelet factor 3. By using PNH rather than normal platelets, a further 4- to 230-fold increase in sensitivity for antibody detection was achieved (Table II, Fig. 2). It seems probable, therefore, that PNH platelets will be useful for studies of certain platelet-antiplatelet systems as evidenced by our preliminary observation that they can be used to detect "autoantibody" in the sera of some patients with idiopathic thrombocytopenic purpura (ITP) (1). Papain treatment of normal platelets provides still a further increase in antibody sensitivity, up to 2000-fold greater than that of normal platelets (Fig. 2), and it is tempting to speculate that enzymatically treated platelets, which carry on their surface the major histocompatibility antigens (50, 51), may prove useful for the early detection of antibodies associated with transplant rejection.

The possible advantages of other isotopic labels such as 5-hydroxytryptamine as indicators for platelet lysis also require exploration, and it is likely that modifications in the technique described here will be forthcoming. It seems very probable, however, that release of isotopes from labeled platelets will provide a useful tool for the study of platelet-antiplatelet immune systems and perhaps for more general studies of the mechanism of immune cytolysis.

APPENDIX

Calculation of the relative contributions of "immunologic" and "spontaneous" processes to total platelet lysis. If the probabilities of a platelet being lysed immunologically and spontaneously are y and b respectively, then the probability of a platelet not being lysed by one or the other of these processes is $(1-y)$ or $(1-b)$. If the two lysis mechanisms are independent of one another, the probability of a platelet escaping lysis by either of these processes is then the product of the two probabilities $(1-y)(1-b)$. The value b was determined by dividing the fraction of total ^{51}Cr released in control tubes containing no antibody by 0.8, and the value y, representing the fraction of cells lysed immunologically in experimental tubes, was calculated from the equation:

$(1-y\ (1-b) =$ fraction of platelets not lysed $= 1-F$,
where $F =$ fraction of total ^{51}Cr released $\div 0.8$.
Solving for y: $y = F - b/1 - b$.

It is apparent that when 80% of ^{51}Cr is released, $y = 1.0$ or 100%, and when ^{51}Cr released equals spontaneous lysis (b) in control tubes, $y = 0$. The validity of this calculation, of course, depends upon the reasonable but as yet unproved assumption that immunologic lysis and "spontaneous" lysis of platelets are independent processes. Given this assumption, the above relationship is valid even if "spontaneous" and "immunologic" lysis operate through similar mechanisms.

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