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Abnormal Solubilization by Triton X-100 of Erythrocyte Membranes from Patients with Paroxysmal Nocturnal Hemoglobinuria[1] (38325)

AGUSTIN P. DALMASSO, MARIA C. PIZZIMENTI, ELSA VACS, AND ALBERTO DIAZ[2]

Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Veterans Administration Hospital, Minneapolis, Minnesota 55417; and Institute of Medical Research and Department of Clinical Laboratories, University of Buenos Aires, Argentina

Paroxysmal nocturnal hemoglobinuria (PNH) is a hematological disorder characterized by an intrinsic abnormality of the erythrocyte, leukocyte and platelet plasma membranes (1, 2). The erythrocytes may be remarkably susceptible to the hemolytic action of complement and the patients may have one to three populations of erythrocytes which differ in susceptibility to lysis by complement (3). Several approaches have been taken to delineate the characteristics of the membrane defect in PNH but its nature still remains enigmatic. Although the data on possible alterations in chemical composition appear controversial (1, 4, 5), a severe reduction in acetylcholinesterase activity is well documented (6). Normal erythrocytes treated with certain proteolytic enzymes (7) or sulfhydryl reagents (8–10) have many features of the PNH red cells, especially an increased susceptibility to complement lysis at slightly acid pH.

The purpose of this paper is to report a new property of the PNH erythrocyte membrane, namely an abnormality in the solubilization of membrane components caused by the nonionic detergent Triton X-100. A portion of this work was reported previously in abstract form (11).

Materials and Methods. PNH patients and controls. The studies were carried out on red blood cell membranes from 8 adult PNH patients who had positive acidified serum lysis (12) and sugar water (13) tests. At the time of the study the red cells from the patients yielded 17–40% hemolysis in the acidified serum test and the reticulocyte counts varied from 1.4 to 11.0%. Four of the patients had never been transfused. The other four patients were transfused repeatedly with red cells but received no transfusion for at least 3 mo prior to these studies.

Controls consisted of erythrocyte membranes from four adult patients with hemolytic anemias who had negative acidified serum and sugar water hemolysis tests, as follows: autoimmune hemolytic anemia associated with systemic lupus erythematosus (4.7% reticulocytes), hereditary spherocytosis (7% reticulocytes), a form of dyserythropoietic anemia (3% reticulocytes) and an ill-defined hemolytic anemia with 17% reticulocytes. Control studies were also performed with erythrocyte membranes from blood bank donors that were matched with patients' age as well as ABO and Rh (D) blood group types.

Preparation and solubilization of erythrocyte membranes. Venous blood was obtained in ACD anticoagulant and the plasma and buffy coat were removed after centrifugation. The erythrocytes were washed 3 times with 0.11 *M* phosphate buffer, pH 7.4, and then lysed with 40 volumes of 5 m*M* phosphate buffer, pH 7.4, per volume of packed erythrocytes (14). The membranes were washed 3 times with this buffer and recovered by centrifugation at 30,000*g* for 20 min. The entire procedure was carried out at 2–4°.

In experiments involving immunoelectrophoresis and immunodiffusion, packed erythrocyte membranes were obtained by sedimentation at 30,000*g* for 20 min and adjusted to a concentration of 5–8 mg protein/ml with the 5 m*M* phosphate buffer. They were then kept at 4–6° until subjected to solubilization and immunological analysis, which was done a few

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[2] Research Fellow of the National Research Council of Argentina.

hours after preparation of the membranes and again 4–5 days later. Solubilization was performed with Triton X-100 (Rohm and Haas Co., Philadelphia) at a final concentration of 1–5% (vol/vol). The reaction mixture was held at 24° for 20 min and then subjected to the analytical procedures.

For studies of degree of solubilization of membrane constituents, the packed membranes were suspended in the 5 m*M* phosphate buffer to yield an optical density of 0.5 at 550 nm. Samples of membrane suspension were incubated for 1 hr at 24° with 3.1 and 15.5 m*M* Triton X-100. After centrifugation at 100,000*g* for 1 hr, the supernatants were analyzed chemically to evaluate the degree of solubilization of membrane constituents.

Antisera against erythrocyte membranes. Washed membranes were centrifuged at 30,000*g* for 20 min and 1 ml of the pellet containing 5–10 mg protein was incorporated in Freund's complete adjuvant and injected into a rabbit, intramuscularly and in the foot pads. Three to 4 weeks later a second injection of membranes in complete Freund's adjuvant was given at multiple subcutaneous and intramuscular sites. Four weeks later booster injections of membranes, usually without adjuvant and occasionally with incomplete Freund's adjuvant, were given subcutaneously every week until the antisera were satisfactory, as evaluated immunoelectrophoretically with Triton-solubilized normal membranes. Six anti-normal membrane antisera were prepared. One was against type A, Rh (D) positive membranes and another against type O, Rh (D) positive membranes. Four antisera were obtained against mixtures of normal membranes from the various types of the ABO system. An antiserum was prepared by immunization with membranes from 3 PNH patients. The antisera were absorbed with normal human serum until no reaction with human serum could be detected by immunoelectrophoresis and double immunodiffusion.

Antisera were also prepared against a β_2 glycolipoprotein membrane component that was purified by preparative electrophoresis of normal membranes solubilized with 2.5% Triton X-100 (15). Upon immunoelectrophoresis against a potent anti-membrane antiserum, this preparation yielded a single β_2 line. The glycolipoprotein used in each injection contained 1 mg of hexose and was mixed with 2.5% Triton X-100 and incorporated into complete Freunds's adjuvant. Each of 2 rabbits received 3 injections with intervals of 2 and 1 mo. The glycolipoprotein was isolated from B type membranes for the first injection, from type O for the second, and from type AB for the third. The animals were bled 2 weeks after the last injection. Upon testing with unfractionated membranes solubilized with Triton X-100, a single line was obtained by Ouchterlony and immunoelectrophoresis analysis.

Immunological and chemical procedures. Immunoelectrophoresis (16) was carried out in 1% agar. The position of precipitin lines of membrane components was assigned according to that of known serum proteins, as indicated by concomitant immunoelectrophoresis of normal human serum. Double immunodiffusion was performed according to Ouchterlony (17). Standard methods were used to measure protein (18), hexoses (19), cholesterol (20), and phospholipids (21).

Polyacrylamide gel electrophoresis. This was carried out in the presence of sodium dodecyl sulphate, using two different techniques (22, 23). Gels were stained with coomassie blue for protein and with a periodic acid Schiff procedure for carbohydrate (23).

Susceptibility of PNH and normal erythrocytes to hemolysis by Triton X-100. One-tenth ml of a 10% suspension of erythrocytes in Veronal buffer, pH 7.4, containing 142 m*M* NaCl (18), was added to 1 ml of the same buffer or to 1 ml of Veronal buffer containing 129 m*M* KCl and 13 m*M* NaCl. To each tube was added 25 μl of an aqueous solution of Triton X-100 at various concentrations to yield 30–90% hemolysis with normal erythrocytes. The percentage of hemolysis was obtained by comparison to a 100% lysis tube which was prepared by mixing 0.1 ml of the erythrocyte suspension with 1.025 ml of distilled water. Blanks consisted of cells that were incubated with the Veronal buffers, without addition of Triton X-100. All mixtures were incubated for 1 hr at 37° with frequent mixing. The unlysed cells were removed by centrifugation and the degree of lysis was evaluated photocolorimetrically at 541 nm. The small amounts of Triton X-100 that were used in the experimental tubes caused no interference with this step in the quantitation of lysis.

Results. Immunological properties of PNH erythrocyte membranes solubilized with Triton

X-100. PNH and normal membranes were treated with 1, 2.5, and 5% Triton X-100 and subjected to immunoelectrophoresis with potent anti-membrane antisera. A typical result (Fig. 1) demonstrated that the main difference between the two types of membranes pertains to a β_2 line that was readily apparent with normal membranes but was either very faint or absent with PNH membranes. Many preparations from 8 PNH patients were examined over a 2-year period. The studies usually showed no β_2 line with 1 and 2.5% Triton X-100 and a weak β_2 line with 5% Triton X-100. In few instances, however, a weak β_2 line could be demonstrated with 2.5% Triton X-100. Membranes from 20 normal controls and 4 patients with hemolytic anemias other than PNH regularly developed the β_2 immunoelectrophoretic line after solubilization with 1–5% Triton X-100. This behavior was observed with all anti-membrane antisera employed, including an antiserum against PNH membranes, regardless of whether membranes and antisera were matched for ABO and MN blood group specificities.

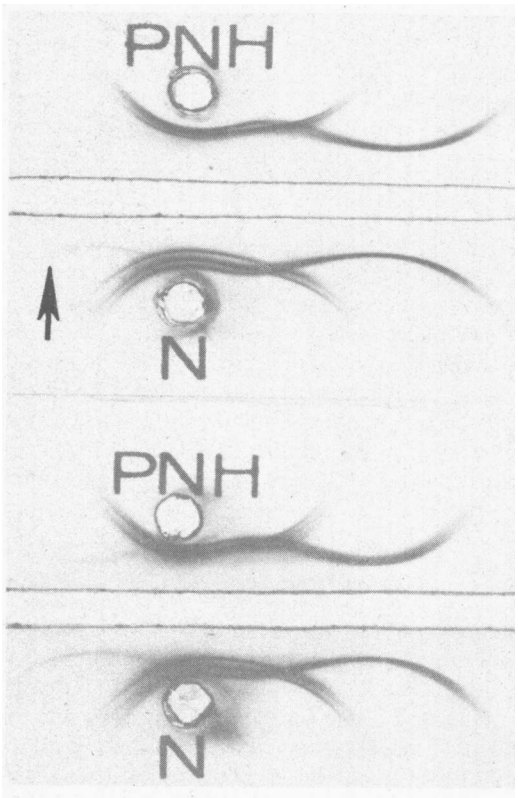

FIG. 1. Immunoelectrophoresis analysis of paroxysma nocturnal hemoglobinuria (PNH) and normal (N) red blood cell membranes solubilized with 2.5% (upper panel) or 5% (lower panel) Triton X-100. Anode was to the right. Arrow indicates β_2 component. In this experiment the purified membranes were maintained for 5 days at 4–8° before use, to facilitate the development of the α_1 component; these results were otherwise identical with those obtained when immunoelectrophoresis was performed immediately after preparation of the membranes.

It was considered possible that the abnormal PNH membrane component was not visible at low Triton X-100 concentrations because it might occupy an anomalous position in the α–β_1 area, where it could not be seen because of the complexity of the pattern in this region. However, this possibility was excluded by immunoelectrophoresis experiments with specific antisera to the glycolipoprotein responsible for the development of the β_2 line with normal membranes, which demonstrated the same deficiency in the PNH membranes as obtained with anti-whole membrane antisera.

Another difference in the immunoelectrophoretic patterns of PNH and normal membranes was that the three distinctly separated arcs obtained in the β_1 area with normal membranes could not be seen clearly separated with PNH membranes, particularly with the lower concentrations of Triton X-100 (Fig. 1).

Upon double immunodiffusion analysis with anti-membrane antisera, PNH and normal membranes solubilized with 1–5% Triton X-100 yielded four lines of precipitation which resulted in reactions of complete identity (Fig. 2). Although with PNH membranes the two lines near the antigen well usually differed in diffusion characteristics from the homologous lines obtained with normal membranes, a consistent pattern could not be established. The line closest to the antiserum well, which corresponds to the immunoelectrophoretic β_2 line (unpublished observations), appeared regularly with both PNH and normal membranes. Reactions of complete identity were obtained when anti-PNH and anti-normal membrane antisera were examined by double immunodiffusion with either PNH or normal membranes.

SDS-polyacrylamide gel electrophoresis showed no consistent differences in the protein and carbohydrate patterns of PNH and normal erythrocyte membranes unexposed to Triton X-100. However, by using a similar approach, abnormalities in erythrocyte membranes from PNH patients have recently been demonstrated

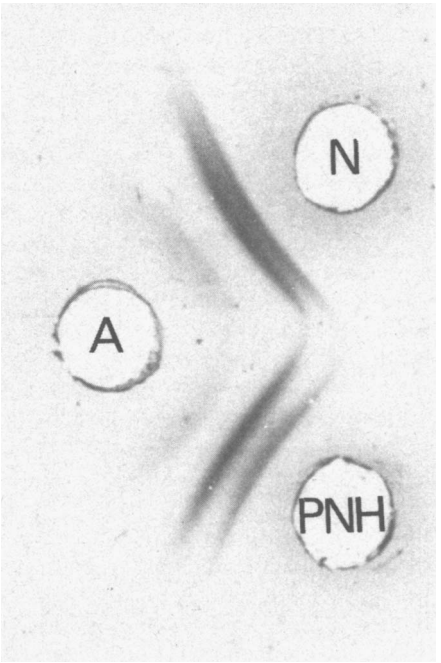

FIG. 2. Double immunodiffusion analysis of paroxysmal nocturnal hemoglobinuria (PNH) and normal (N) erythrocyte membranes solubilized with 2.5% Triton X-100. A: antiserum against normal human erythrocyte membranes. Identical results were obtained with an antiserum against PNH erythrocyte membranes.

by others (24, 25).

Degree of solubilization of PNH erythrocyte membrane constituents by Triton X-100. Measurements were performed of the amounts of protein, cholesterol, and hexose that were dissociated from the membranes of PNH patients and normal controls by treatment with Triton X-100. Table I indicates that this detergent produced a significantly lower degree of solubilization of cholesterol and hexose with PNH than with normal membranes. However, an identical amount of protein was dissociated from both cell types. Table I also demonstrates that PNH and normal membranes contained identical total amounts of protein, cholesterol and hexose. These results demonstrate that the Triton-mediated solubilization of cholesterol and hexoses with PNH membranes was 18–27% lower than with normal membranes. The erythrocyte membranes from the four patients with hemolytic anemias other than PNH were studied as above and found not to differ from the membranes of normal donors in susceptibility to solubilization by Triton X-100.

The possibility was considered that the apparent low degree of solubilization by Triton X-100 of PNH erythrocyte membrane cholesterol and hexoses might be reflecting a larger loss of membrane components in PNH than in normal membranes during the washing steps involved in the preparation procedure. However, the following experiment demonstrates that PNH and normal membranes undergo similar degrees of loss of membrane components during the washing steps. Analyses were performed on the buffers used in the second and third washes of the intact erythrocytes and in the second and third washes of the membranes recovered after osmotic lysis. Cholesterol was assayed on extracts (26) of the unconcentrated buffers, but the latter were concentrated 100 times by ultrafiltration to perform quantitation of hexoses and tests of immunologically reactive membrane components. The following results were obtained with membranes from two normal controls and one PNH patient (percent of total membrane content removed during the four washing steps). Cholesterol: controls, 12 and 9; PNH, 8. Hexoses: controls, 7 and 4; PNH, 5. Ouchterlony and immunoelectrophoresis analyses revealed no precipitating membrane component in the washing buffers. These experiments demonstrate that during the washing steps the loss of membrane cholesterol and hexoses was of similar degree in PNH and normal membranes. Therefore, the observation of an abnormal solubilization of PNH membrane constituents by Triton X-100 is a real phenomenon and not the consequence of an artifact developed during the membrane preparation procedures.

Susceptibility of PNH red blood cells to the hemolytic effect of Triton X-100. The possibility was considered that the abnormality in the solubilization of PNH erythrocyte membrane constituents by Triton X-100 might be related to an altered susceptibility of the intact PNH erythrocyte to the hemolytic effect of the detergent. This problem had to be explored with amounts of Triton X-100 much smaller than those employed in the solubilization studies. Because the susceptibility of normal cells to lysis by Triton X-100 is greatly affected by the monovalent cations present in the reaction system (27), experiments were carried out using a buffer with a high Na content and another with a high K content. The results (Table II) indicated a similar susceptibil-

TABLE I. Chemical Composition and Degree of Solubilization by Triton X-100 of Erythrocyte Membranes from Patients with Paroxysmal Nocturnal Hemoglobinuria.

Membrane constituent	Membranes from	Total content, μg/ml membrane suspension	% Membrane constituent[a] solubilized with	
			3.1 m*M* Triton	15.5 m*M* Triton
Protein	PNH patients	1,332 ± 212	53.0 ± 12.6	63.1 ± 8.4
	Normal controls	1,345 ± 198	53.5 ± 12.7	62.0 ± 9.8
	P value	> 0.4	> 0.4	> 0.4
Cholesterol	PNH patients	168 ± 47	33.1 ± 5.2	62.3 ± 9.7
	Normal controls	172 ± 49	45.3 ± 6.0	75.8 ± 12.1
	P value	> 0.4	< 0.01	0.05
Hexose	PNH patients	120 ± 11	36.9 ± 2.5	51.1 ± 4.8
	Normal controls	122 ± 22	45.6 ± 4.4	64.6 ± 8.9
	P value	> 0.2	< 0.005	< 0.02

[a] Results of cholesterol and hexose measurements represent mean ± SEM of six determinations on membranes from five PNH patients and six normal controls. Results of protein measurements represent mean ± SEM of four determinations on membranes from four cases in each group.

ity of PNH and normal red blood cells to hemolysis by Triton X-100.

Discussion. The immunoelectrophoresis studies revealed a deficiency of the PNH erythrocyte membranes to yield a β_2 line after solubilization with concentrations of Triton X-100 (1–5%, *i.e.*, 16–80 m*M*) that consistently produced this line when used with normal membranes. The membrane component responsible for the β_2 line could be clearly seen, however, by immunodiffusion analysis of PNH membranes solubilized with 1–5% Triton X-100, which was probably due to the greater sensitivity of this technique. The nature of the component of normal membranes responsible for production of the β_2 line has been characterized previously (15) as a macromolecular glycolipoprotein similar to the low density fraction (fraction *K*) described earlier by Morgan and Hanahan (28). It contains little protein but is rich in lipids and hexoses, and its antigenic determinants are exposed on the external surface of the erythrocyte membrane. It has high affinity for Triton X-100, being retained firmly in the micellar arrangement of the detergent (15). Therefore, this suggests that the abnormality in the development of the β_2 line with PNH membranes reflects an anomalous reactivity of the corresponding glycolipoprotein with Triton X-100. It is of interest that even with normal membranes, critical ratios of the membrane glycolipoprotein to detergent are required for the development of the β_2 line (unpublished observation). In aqueous solutions, concentrations of Triton X-100 exceeding the critical micellar concentration form micelles consisting of 100–230 monomers with

TABLE II. Degree of Hemolysis Caused by Triton X-100 Upon Red Blood Cells from Patients with Paroxysmal Nocturnal Hemoglobinuria.

Triton concentration ($\times 10^{-5}$ *M*)	Cations present during reaction ($\times 10^{-3}$ *M*)	% Hemolysis (mean & range[a])	
		PNH	Normal controls
7.7		59 (50–65)	67 (51–77)
8.5	Na, 142	78 (77–79)	79 (73–86)
9.6		86 (82–89)	87 (86–88)
6.3		54 (52–57)	48 (33–65)
7.0	K, 129; Na 13	75 (71–77)	72 (56–83)
7.7		85 (79–89)	82 (76–87)

[a] Results represent three experiments with red cells from three cases in each group.

micellar molecular weights of 60,000–160,000 (29, 30). It should be possible, therefore, to explore the mode of interaction of Triton X-100 with the membrane glycolipoprotein from PNH and normal membranes, including size, shape, and stability of the detergent-membrane component complex.

A defect in the solubilization of PNH membranes was also shown with concentrations of Triton X-100 that yielded partial solubilization of membrane components. It was found that hexoses and cholesterol dissociated to a lesser extent from PNH than from normal membranes. However, identical amounts of protein were dissociated from both types of membranes. Because only limited amounts of membranes were available from PNH patients, it was not possible to quantitate the degree of dissociation by Triton X-100 of other membrane constituents. The possibility that the abnormality in Triton-mediated solubilization of PNH membranes might be related to the presence of a large population of young red cells was eliminated by the results with red cells from patients with hemolytic anemias other than PNH. These membranes behaved like normal membranes with regard to (a) the degree of solubilization of membrane constituents by concentrations of Triton X-100 causing partial membrane solubilization, and (b) the production of a normal immunoelectrophoretic pattern after solubilization with Triton X-100.

Obviously, a clear definition of the membrane defect responsible for the abnormality in Triton-mediated solubilization would be of great interest. However, this may be difficult to achieve because, among other reasons, the PNH erythrocytes are heterogeneous, as demonstrated by the existence in individual patients of erythrocyte populations that differ in susceptibility to complement-mediated lysis (3) as well as in content of acetylcholinesterase activity (31). The studies reported here indicate that the organization of the erythrocyte membrane components is abnormal in PNH and that the fundamental defect of the PNH erythrocyte membrane may reside in a membrane glycolipoprotein with high affinity for Triton X-100. It remains to be established whether there is any direct relationship between the anomalous reactivity with Triton X-100 and the two important features of the PNH erythrocyte membrane, namely the high susceptibility to complement-mediated lysis and the low content of acetylcholinesterase activity.

Summary. Erythrocyte membranes from patients with paroxysmal nocturnal hemoglobinuria (PNH) were found to undergo a deficient degree of solubilization when reacted with the nonionic detergent Triton X-100. Upon exposure to concentrations of Triton X-100 causing partial membrane solubilization in 5 m*M* phosphate buffer, pH 7.4, cholesterol and hexoses from patients' membranes dissociated to a lower degree than the membrane constituents from normal controls and patients with hemolytic anemias unrelated to PNH. The degree of solubilization of membrane protein was identical with all types of erythrocytes. The degree of hemolysis caused by Triton X-100 was similar with PNH and normal red cells. Immunoelectrophoresis of normal membranes solubilized with 1–5% Triton X-100 yielded several α–β_1 lines and one β_2 line. PNH membranes produced similar α–β_1 lines but the β_2 line was absent or very faint. Since it was known that the β_2 component of normal membranes is a glycolipoprotein with high affinity for Triton X-100, the immunoelectrophoretic abnormality of PNH erythrocyte membranes indicates an altered reactivity with Triton X-100 of this glycolipoprotein membrane component. The findings suggest that in PNH there is a major defect in the organization of the erythrocyte membrane constituents which is mediated by an as yet undefined anomaly in the membrane β_2 glycolipoprotein.

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