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Complement-Mediated Lysis in Different Cationic Media of Erythrocytes from Patients with Paroxysmal Nocturnal Hemoglobinuria (37780)

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The main feature of the red cells from patients with paroxysmal nocturnal hemoglobinuria (PNH) is an increased susceptibility to lysis by complement (C) (1–3), but the nature of the membrane defect underlying the high reactivity with C is unknown. The studies on susceptibility of PNH erythrocytes to lysis by C have usually been carried out in media containing Na as the main cation. It is known, however, that the degree of lysis of normal human erythrocytes depends on the alkali metal cation that is present during the C reaction (4, 5). Thus, under conditions of limited amounts of C in the reaction system, the degree of lysis in 145 m*M* KCl was about three times as large as in NaCl. Intermediate amounts of lysis were obtained with Rb, Li, and Cs, resulting in the following selectivity series: K > Rb > Li > Cs > Na. It was found that K, Rb, and Cs modify immune lysis through an effect on the final stage of the C reaction. Thus, when normal erythrocytes were treated in Na buffer with concentrations of C that yielded 20–50% lysis and then the unlysed cells were washed and transferred to media containing the other monovalent cations, additional lysis occurred in K, Rb, and Cs but not in Na and Li (4, 6). Therefore, it was concluded that there is an important effect of the alkali metal cations on the E*, the cell that has been damaged by C but has not as yet undergone lysis (4, 6). The effect of Li and Cs on lysis of E*, however, was different from their effect on the whole C sequence. The cationic series obtained with E* was K = Rb = Cs >> Li = Na (4). In view of the significant role of alkali metal cations upon the degree of immune lysis of normal erythrocytes, we decided to explore the role of these ions in C-mediated lysis of PNH erythrocytes.

Materials and Methods. Erythrocytes. Blood was collected in ACD anticoagulant, stored at 4°, and used within 3 days. Three types of human erythrocytes were used in this study: PNH, PNH-like, and normals. The PNH erythrocytes were obtained from five adult patients who had positive acidified serum lysis (7) and sugar water (8) tests. At the time of the study, the red cells of the various patients yielded from 13 to 38% lysis in the acidified serum test, and the reticulocyte counts varied from 1.4 to 10%. Three of the patients had never been transfused. Two patients were repeatedly transfused with red cells but received no transfusion for at least 3 months prior to these studies. Four of the patients were type O, Rh (D) positive, and one was type A, Rh (D) positive. Normal, type O, Rh positive erythrocytes were obtained from blood donors. Artificial PNH cells (PNH-like) were prepared by treatment of normal red cells with 0.635 *M* reduced glutathione, as previously described (9).

Immune lysis. A rabbit antiserum against type O, Rh positive erythrocyte membranes (5, 10) was employed. It was subjected to 56° for 30 min before use in immune lysis. The erythrocytes were washed three times with veronal buffer containing 145 m*M* NaCl,

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0.15 m*M* $CaCl_2$, and 0.5 m*M* $MgCl_2$, pH 7.3 (11). This buffer is referred to as Na buffer. The erythrocytes were suspended in Na buffer at a concentration of 10^9 cells/ml. One milliliter of the erythrocyte suspension was mixed with 960 μl of Na buffer and 40 μl of anti-membrane antiserum, which corresponded to 2 hemolytic units of antibody (11). The mixture was incubated at 37° for 30 min. The following steps were carried out at 0–2°. Sensitized erythrocytes were washed three times with Na buffer and resuspended at a concentration of 5×10^8 cells/ml. Four tenths milliliter were transferred to each of several tubes and centrifuged, and the supernatants were discarded. Then the erythrocytes were suspended in 0.8 ml of Na buffer or of buffers with identical composition to the Na buffer except that 145 m*M* of the chloride salts of K, Rb, Li, or Cs were substituted for NaCl. These solutions are referred to as K buffer, Rb buffer, etc. Finally, 0.2 ml of diluted fresh human serum was added to furnish C. The serum was first diluted 1:10 in Na buffer and then further diluted with the buffer used for resuspending the red cells. C was used at final dilutions that yielded 17–50% lysis in Na buffer, as follows: with normal red cells, 1:150; with PNH and PNH-like cells, from 1:150 to 1:300. Incubation was carried out at 37° for 1 hr with intermittent manual mixing. The C reaction was terminated by addition of 2 ml ice-cold buffer of the same composition as used for immune hemolysis. Blanks were identical to the experimental reaction mixtures except that C was omitted. The degree of lysis was calculated from photocolorimetric determinations at 540 nm of the released hemoglobin after centrifugation of the unlysed cells.

Acidified serum lysis test. Each sample of PNH erythrocytes was subjected first to the acidified serum test according to a routine procedure (7). Then, to explore the effect of alkali cations on the acid lysis test, the following method was used. The red cells were washed three times with Na buffer and centrifuged at 2500*g* for 10 min. Twenty-five microliters of packed cells were added to tubes, each containing 0.3 ml of one of the buffers described above, 0.2 ml of fresh, ABO-compatible human serum, and 50 μl of 0.1 *N* HCl. The pH of the reaction mixture was 6.8. Controls included a tube without HCl and a tube with 0.2 ml of human serum heated at 56° for 30 min instead of fresh serum. The reaction mixtures were incubated at 37° for 1 hr and then the degree of lysis was determined as above. Normal erythrocytes that were subjected to similar treatment served as negative controls.

Preparation and lysis of E.* A portion of the red cells that remain unlysed after treatment in Na buffer with antibody and limited amounts of C (yielding 20–50% lysis in 1 hr at 37°) will undergo lysis if transferred and incubated in buffers with certain alkali metal cations (4, 6). These cells are referred to as E* in this publication. They were prepared by reacting in Na buffer 2×10^9 sensitized normal erythrocytes with sufficient human C (final dilution, 1:150) to yield 20–50% lysis upon incubation for 1 hr at 37° in a total volume of 10 ml. The residual, C-treated, unlysed cells were washed three times at 0–4° with large volumes of Na buffer, divided in samples of 10^8 cells which were then resuspended in 1 ml of the various alkali metal cation buffers, and incubated at 37° for 1 hr. Finally, the degree of lysis was determined as above.

Results. In the first group of experiments, PNH, PNH-like, and normal human erythrocytes were compared in the susceptibility to lysis by antibody and C (immune lysis) in different media containing the various alkali metal cations. As summarized in Table I, the effect of the monovalent cations was similar with the three types of red cells, yielding a selectivity series in which $K \geqq Rb > Li > Cs \geqq Na$. It was difficult to adjust the C concentrations to obtain the same degree of lysis in Na buffer with all cell types. Thus, the degree of lysis of PNH cells was larger than that of normal cells in Na and Cs buffers but not in the other cations. Consequently, when the results were presented as percentage of change in degree of lysis with respect to the amount of lysis obtained in Na (Fig. 1) it appeared that K, Rb, and Li caused a larger enhancement of immune lysis of normal than of PNH cells. Because of the rela-

TABLE I. Effect of Alkali Metal Cations on Lysis by Antibody and Complement of Red Cells from Patients with Paroxysmal Nocturnal Hemoglobinuria and of Normal Red Cells Artificially Converted into PNH-like Cells.

Type of red cell	Number of experiments	% Lysis in (Mean ± SE)[a]				
		Na	K	Li	Rb	Cs
PNH	6	31 ± 3.5	50 ± 3.8	46 ± 5.0	49 ± 3.9	32 ± 3.4
PNH-like	2	33 (23,43)[b]	54 (45,63)	51 (43,59)	55 (47,63)	36 (24,46)
Normal	9	22 ± 2.0	57 ± 3.6	47 ± 3.4	55 ± 2.7	27 ± 3.0

[a]Statistical analysis (Student t test). PNH red cells: Na vs K, Na vs Li, Na vs Rb, K vs Cs, Li vs Cs, and Rb vs Cs, $P < 0.02$; Na vs Cs, K vs Li, K vs Rb, and Li vs Rb, not significant ($P > 0.05$). Normal red cells: Na vs K, Na vs Li, Na vs Rb, K vs Li, K vs Cs, Li vs Rb, Li vs Cs, and Rb vs Cs, $P < 0.05$; Na vs Cs, and K vs Rb, not significant.

[b]Figures in parentheses represent individual experiments.

tively large degree of lysis obtained in Na buffer with patient cells (Table I), it was felt that in this experiment we might have examined the effect of alkali cations not only on the C-sensitive red cell population, but also on the less abnormal, C-insensitive population (12). Therefore, an experiment was carried out with cells from two patients using concentrations of C yielding less than 10% lysis in Na buffer. Under these conditions, the degree of immune lysis in the various monovalent cations continued to be enhanced by $K \geqq Rb > Li > Cs \geqq Na$.

Representative experiments on the effect of alkali cations on acidified serum lysis of PNH and PNH-like human erythrocytes are presented in Table II. It can be seen that in most cases there was enhancement of lysis by K, Rb, and Cs, but not by Li (experiments 1 and 2 with PNH cells and experiments 4 and 5 with PNH-like cells). In other cases, however, there was little or no

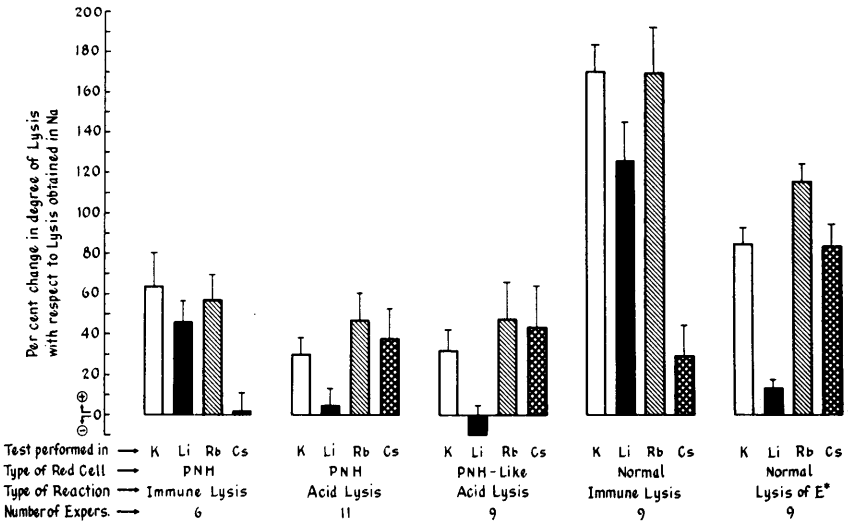

FIG. 1. Effect of alkali metal cations on the degree of complement-mediated lysis of PNH, PNH-like, and normal human erythrocytes. The various types of complement-mediated reactions are described in Materials and Methods. Vertical lines indicate SEM. Statistical analysis: Combinations yielding statistically significant differences ($P < 0.05$): PNH cells, immune lysis: Cs vs K, Li, and Rb; PNH cells, acid lysis: Li vs K, Rb, and Cs; PNH-like cells, acid lysis: Li vs K, Rb, and Cs; normal cells, immune lysis: Cs vs K, Li, and Rb; K vs Li; normal cells, lysis of E*: Li vs K, Rb, and Cs; Rb vs K, and Cs. In all other combinations the differences were not significant.

TABLE II. Acidified Serum Lysis Test in Different Cationic Media of Red Cells from Patients with Paroxysmal Nocturnal Hemoglobinuria and of Normal Red Cells Artificially Converted into PNH-like Cells.

Type of red cell	No. of experiment	% Lysis in				
		Na	K	Li	Rb	Cs
PNH	1	6	10	4	15	16
	2	24	27	21	28	28
	3	17	20	20	23	20
PNH-like	4	9	18	7	24	23
	5	22	29	20	29	27
	6	17	21	18	20	21

difference in the degree of lysis obtained in the various alkali metal cations (experiments 3 and 6). Results of a larger series of experiments on acidified serum lysis of naturally occurring PNH cells and PNH-like cells are presented in Fig. 1 as percentage of change in degree of lysis in the various cations with respect to the amount of lysis obtained in Na. It can be seen that the pattern of changes in acidified serum lysis was markedly different from that obtained in immune lysis by antibody and C. In acid lysis, K, Rb, and Cs, but not Li, caused enhancement of lysis. Figure 1 also demonstrates that the pattern obtained in acidified serum lysis of PNH cells was similar to that obtained in lysis of E* prepared by exposure of sensitized normal erythrocytes to limited amounts of C in Na buffer and then transferred and incubated in the various alkali metal cations, in C-free media.

Discussion. The red cells of PNH patients are known to be generally more susceptible to C-mediated lysis than normal red cells (1–3). The recent study of Rosse (12) demonstrated that patients with PNH may have one to three populations of erythrocytes which differ in susceptibility to lysis by C and that the relative amounts of C-sensitive and C-insensitive erythrocytes differ widely among various patients. Since it had been established that the degree of C-mediated lysis of normal human erythrocytes is influenced by the alakli metal cations present in the reaction system (4, 5), it became of interest to explore the response of the PNH cells to C in various cationic media. The results presented here indicate that the alkali metal cations modify the degree of C-mediated lysis of PNH erythrocytes in two different manners which are characteristic for each of the two types of C reactions investigated. Thus, when the cells were treated with antibody and C (immune lysis), the monovalent cations promoted lysis as follows: $K \geqq Rb > Li > Cs \geqq Na$. However, when the cells were subjected to the acidified serum lytic reaction, K, Rb, and Cs facilitated hemolysis, but Li had no effect. The main difference between these patterns is a change in the effects of Li and Cs in the two experimental situations.

A comparative study of the effect of alkali metal cations on immune lysis of PNH and normal cells demonstrated qualitatively similar responses. The experiments were performed with an antiserum prepared in rabbits by immunization with purified human erythrocyte membranes and human serum as the source of C. These results were comparable to those reported previously (5) with normal human erythrocytes sensitized with a similar antiserum but subjected to guinea pig C.

The influence of the various alkali metal cations on acidified serum lysis of PNH cells was similar to that upon lysis of normal human E*. In the latter situation, the sensitized erythrocytes were suspended in Na buffer and subjected to limited amounts of C at 37° for 1 hr. Then the unlysed cells were washed in Na buffer and transferred and incubated in the various alkali metal cations. This resulted in additional lysis with K, Rb, and Cs, but not with Li and Na. This phenomenon was observed previously with human erythrocytes reacted with guinea pig C (4) and was corroborated in the studies reported here using human C. It is of interest that pretreatment of HK sheep erythrocytes with alkali metal cations modified the degree of immune lysis carried out in Na buffer, resulting in a pattern similar to that of E* or acid hemolysis of PNH cells (13). Immune lysis of HK erythrocytes from a different group of sheep, however, was not affected by preincubation with alkali metal cations (5).

It has been shown previously that while the acidified serum lysis reaction is mediated by activation of the alternate C pathway (14–16), immune lysis of normal cells by antimembrane antibody and C depends primarily on activation of the classical pathway (17, 18). Therefore, in view of our findings it seems adequate to propose, first, that the pattern of cationic influence on immune lysis in which $K \geqq Rb > Li > Cs \geqq Na$ results from the effects of the monovalent cations on the classical mechanism of C, and second, that the pattern $K = Rb = Cs >> Li = Na$ results from the effect of the cations on the final stage of immune lysis and is also apparent in situations of predominance of the alternate C pathway.

Summary. A study was performed of the effects of alkali metal cations on the degree of complement (C)-mediated lysis of red blood cells from patients with paroxysmal nocturnal hemoglobinuria (PNH) as well as of artifically prepared, PNH-like red cells. When rabbit antiserum and human C were used, the alkali metal cations enhanced immune lysis of normal and PNH red cells as follows: $K \geqq Rb > Li > Cs \geqq Na$. In the acidified serum reaction of PNH and PNH-like cells, however, enhancement of lysis occurred with K, Rb, and Cs, but not with Li, as compared to the degree of lysis in Na. The latter pattern of cationic effect was identical with that obtained upon the final stage of immune lysis of normal human red cells by antibody and C.

The results indicate that the influence of the alkali metal cations on the final stage of immune lysis after activation of the classical C pathway is similar to that on the sequence of reactions which forms the alternate pathway. A different pattern of influence of the cations was observed in situations of predominance of the complete reaction sequence which constitutes the classical pathway of C activation.

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