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Detection of Complement Components on Unlysed Erythrocytes from Acid Hemolysis and Thrombin Test Reactions in Paroxysmal Nocturnal Hemoglobinuria *

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As a result of an unidentified red cell defect, erythrocytes from patients with paroxysmal nocturnal hemoglobinuria [1] are susceptible to hemolysis in acidified fresh serum (1). This acid hemolysis reaction is augmented by commercial bovine thrombin (2). The role of serum complement in these PNH hemolytic reactions has not been entirely clear (3). Previous studies from this laboratory, utilizing AH·PNHE and Thr·PNHE, have demonstrated the presence of complement on the surface of these cells (4). The present studies provide a more detailed analysis of the components of complement found on AH·PNHE and Thr·PNHE and compare these findings with the components of complement found on residual, unlysed normal erythrocytes surviving hemolysis in human EAC′ systems (5, 6). The implications of these results with respect to mechanisms of hemolysis in the PNH acid hemolysis and thrombin test reactions will be discussed.

Methods

PNHE and normal human erythrocytes were collected under sterile conditions and either used immediately or stored at 4° C in sterile modified Alsever's solution (*7a*) for no longer than 1 week before use. With such storage the hemolytic susceptibility of PNHE remains unaltered for at least 6 weeks (8). PNH and normal sera were separated from defibrinated blood and stored at −70° C until used. At this temperature PNH hemolytic activity of serum is preserved for at least 11 months (9). Antiserum to pooled human serum (anti-whole serum) was prepared in rabbits. This antiserum agglutinates erythrocytes coated with either γ-globulin or complement. Antiserum to human γ-globulin [2] (anti-γ) was prepared in rabbits. This antiserum agglutinates erythrocytes coated with γ-globulin but not complement-coated erythrocytes, and immunoelectrophoresis (10) with whole human serum results in a single γG-globulin precipitin line. Antiserum to human complement (anti-C′) was prepared according to the method of Klein and Burkholder (11). This antiserum agglutinates complement-coated erythrocytes but not γ-globulin–coated erythrocytes. Immunoelectrophoresis of this material with whole human serum results in two β-globulin bands corresponding to the human complement proteins, β_{1C}-globulin (5, 12) and β_{1E}-globulin (5, 13), described by Müller-Eberhard and co-workers. Specific rabbit antisera to human β_{1C}-globulin and β_{1E}-globulin were kindly supplied by Dr. Müller-Eberhard. Before use in antiglobulin agglutination tests, each of the above antisera was heated at 56° C for 30 minutes and then absorbed successively with washed normal group A, B, and O erythrocytes at 4° C until it no longer agglutinated normal erythrocytes of any blood group (*14a*).

The acid hemolysis reaction mixture contained 0.1 ml of washed, packed erythrocytes and 0.9 ml of normal human serum of compatible blood group. Before

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[1] The following abbreviations will be used in this paper: PNH, paroxysmal nocturnal hemoglobinuria; PNHE, erythrocytes from patients with PNH; AH·PNHE, residual, unlysed PNH erythrocytes surviving the acid hemolysis reaction; Thr·PNHE, residual, unlysed PNH erythrocytes surviving thrombin test hemolysis; EAC′ hemolysis, *in vitro* hemolytic reaction involving erythrocytes (E), antibody (A), and complement (C′); C′1, C′2, C′3, and C′4, the first, second, third, and fourth components of complement; β_{1E}-globulin, human C′4; β_{1C}-globulin, a subcomponent of human C′3.

[2] Poliomyelitis immune globulin, Parke-Davis, Detroit, Mich.

mixing, the serum was acidified with 0.3 N hydrochloric acid to the pH range (6.0 to 6.2) giving maximal hemolysis of PNH erythrocytes in this system. The reaction mixture was incubated at 37° C for 15 minutes and centrifuged, and the supernatant was removed for determination of per cent hemolysis. The residual, unlysed erythrocytes were washed three times with saline and subjected to antiglobulin agglutination testing. On a glass tile, 1 drop of a 10 to 15% suspension of erythrocytes in saline was mixed with 1 drop of serial fourfold dilutions of antiserum in saline (14*b*). After 7 minutes incubation, gross agglutination was observed and assigned an arbitrary score of 0 to 4+. The observed agglutination was compared with the reaction of untreated, washed erythrocytes. In addition both treated and untreated erythrocytes were observed for spontaneous agglutination in saline. The procedure for the thrombin test differed from the above only in that 50 NIH U of bovine thrombin [3] in 0.1 ml of saline was added to the acidified serum before incubation with erythrocytes.

Antiglobulin neutralization tests were carried out to determine the ability of certain proteins to neutralize the observed positive antiglobulin reactions with antiwhole serum. In these tests an equal volume of a saline solution of the neutralizing protein was added to a 1:4 dilution of antiwhole serum in saline resulting in a 1:8 final dilution of antiserum. The approximate concentrations of the proteins used in these neutralization studies were γ-globulin,[4] 4 mg per ml; β_{1C}-globulin,[5] 0.2 mg per ml; and β_{1E}-globulin,[5] 0.2 mg per ml. After 30 minutes incubation at room temperature the erythrocyte agglutination reaction of the treated antiserum was compared with that of untreated antiserum similarly diluted 1:8 in saline. Diminution in the agglutination reaction of the treated antiserum would thus indicate the presence of the neutralizing protein on the surface of the erythrocytes tested. Complete inhibition of the reaction would suggest that the neutralizing protein was the sole serum protein on the erythrocyte surface.

Results

Antiglobulin agglutination tests were carried out on untreated PNHE, on AH·PNHE, and on Thr·PNHE in nine PNH patients (Table I). Erythrocytes from all nine were studied with antiwhole serum and anti-γ-globulin. Erythrocytes from seven were studied with anti-C′, anti-β_{1C}-globulin, and anti-β_{1E}-globulin. In no instance was a positive antiglobulin reaction observed with untreated PNHE. After acid hemolysis and thrombin test reactions, however, the AH·PNHE and the Thr·PNHE of all nine patients were agglutinated by antiwhole serum but not by anti-γ-globulin, suggesting that complement coating had occurred (5). Anti-C′ and anti-β_{1C} agglutinated both the AH·PNHE and the Thr·PNHE from all of the seven patients studied. Anti-β_{1E}-globulin likewise agglutinated the Thr·PNHE from all seven but did not agglutinate the AH·PNHE from any of the patients. These results indicate the presence of complement on both AH·PNHE and Thr·PNHE, although anti-β_{1E} agglutination in the systems differed.

TABLE I

*Results of antiglobulin agglutination tests on untreated PNHE and on AH·PNHE and Thr·PNHE**

Antiglobulin serum	No. of patients studied	No. of PNH patients with erythrocytes giving positive antiglobulin agglutination tests		
		Untreated PNHE	AH ·PNHE	Thr ·PNHE
Antiwhole serum	9	0	9	9
Anti-γ-globulin	9	0	0	0
Anti-C′	7	0	7	7
Anti-β_{1C}-globulin	7	0	7	7
Anti-β_{1E}-globulin	7	0	0	7

* PNH, paroxysmal nocturnal hemoglobinuria; PNHE, erythrocytes from patients with PNH; AH·PNHE and Thr·PNHE, residual, unlysed PNHE surviving the acid hemolysis reaction and thrombin test hemolysis, respectively.

Antiglobulin tests on normal erythrocytes before and after acid hemolysis and thrombin test reactions. With normal erythrocytes no hemolysis was observed in the acid hemolysis and thrombin test systems. Table II shows the results of antiglobulin agglutination tests on the erythrocytes of five normal subjects before and after acid hemolysis and thrombin tests, using the same antisera as with the PNHE. The untreated erythro-

TABLE II

Results of antiglobulin agglutination tests on untreated normal erythrocytes and residual unlysed normal erythrocytes from acid hemolysis and thrombin test systems

Antiglobulin serum	No. of subjects studied	No. of normal subjects with erythrocytes giving positive antiglobulin agglutination tests		
		Untreated erythrocytes	Acid hemolysis erythrocytes	Thrombin test erythrocytes
Antiwhole serum	5	0	0	5
Anti-γ-globulin	5	0	0	0
Anti-C′	5	0	0	5
Anti-β_{1C}-globulin	5	0	0	5
Anti-β_{1E}-globulin	5	0	0	5

[3] Upjohn thrombin, kindly supplied by Dr. John T. Correll, Upjohn Co., Kalamazoo, Mich.

[4] Parke-Davis poliomyelitis immune globulin.

[5] These highly purified proteins were kindly supplied by Dr. Müller-Eberhard.

cytes and the acid hemolysis erythrocytes gave negative antiglobulin tests in all five subjects with all antisera. However, the thrombin test erythrocytes of all five were agglutinated by antiwhole serum, anti-C′, anti-β_{1C}, and anti-β_{1E} but not by anti-γ. Thus complement appears to be fixed to normal erythrocytes as well as PNHE in the thrombin test system. In contrast, no complement is fixed to normal erythrocytes in the acid hemolysis system.

Details of the antiglobulin agglutination reactions of AH·PNHE and Thr·PNHE. Table III summarizes the antiglobulin agglutination scores of AH·PNHE and Thr·PNHE obtained from one typical patient, No. 7 (9). In this patient as in the others, Thr·PNHE demonstrated stronger antiglobulin agglutination reactions with antiwhole serum, anti-C′, and anti-β_{1C} than did AH·PNHE with these same antisera. The agglutination of Thr·PNHE by anti-β_{1E} was weaker than with the other antisera. As stated previously, anti-β_{1E} did not react with the AH·PNHE.

Factors influencing the antiglobulin reaction of AH·PNHE and Thr·PNHE. Numerous experiments were carried out to determine the conditions giving a maximal antiglobulin agglutination reaction of AH·PNHE and Thr·PNHE. Changing the concentration of PNHE or increasing the incubation time to 30 minutes produced little change. The single most important factor determining both the degree of hemolysis and the antiglobulin reaction in the acid hemolysis reaction was the pH of the serum added to the system. The results of experiments in an individual patient, No. 3 (9), comparing serum pH, per cent hemolysis, and anti-C′ antiglobulin reaction in acid hemolysis and thrombin test systems are summarized in Table IV. In this patient hemolysis and antiglobulin reaction in the acid hemolysis system were maximal with pH 6.2 serum. In five other patients maximal hemolysis and antiglobulin reactions were observed with serum in the pH range of 6.0 to 6.2, with maximal per cent hemolysis in these patients ranging from 15% to 48%. The pH range of 6.0 to 6.2 is somewhat lower than that reported in the literature for optimal hemolysis in the acid hemolysis reaction (15, 16). This discrepancy may be more apparent than real, since our studies were not carried out in a sealed system to prevent the escape of CO_2 as were these other studies.

TABLE III

Antiglobulin agglutination scores of AH·PNHE and Thr·PNHE of Patient 7*

Antiglobulin serum	AH·PNHE				Thr·PNHE			
	4†	16	64	S	4	16	64	S
Antiwhole serum	2	1	±	0	3	2½	1	0
Anti-γ	0	0	0	0	0	0	0	0
Anti-C′	2	2	1	0	3	3	1½	0
Anti-β_{1C}	1½	1	0	0	3	2	0	0
Anti-β_{1E}	0	0	0	0	1	1	1	0

* 4, maximal agglutination; ±, minimal detectable agglutination; 0, no agglutination; other numbers, intermediate scores.
† 4, 16, and 64 represent reciprocals of dilutions of each antisera. S represents the agglutination reaction of the cells in saline alone.

TABLE IV

Effect of pH on per cent hemolysis and antiglobulin agglutination of unlysed PNH erythrocytes in Patient 3

Serum pH	Acid hemolysis reaction		Thrombin test reaction	
	Per cent hemolysis	AH·PNHE score*	Per cent hemolysis	Thr·PNHE score*
7.4	2.9	.0	40.9	2½
7.0	8.9	±	51.3	2½
6.6	18.5	1	60.2	3
6.2	34.2	2	58.6	3
5.8	30.4	2	58.4	3
5.4	5.8	1	33.4	1½

* Antiglobulin agglutination score using anti-C′ antiserum diluted 1 to 4: 4+, maximal agglutination; ±, minimal detectable agglutination; 0, no agglutination; others, intermediate agglutination.

In contrast with the acid hemolysis reaction there was much less variation in per cent hemolysis and antiglobulin reaction with changes in the serum pH in the thrombin test system. Significant hemolysis and antiglobulin reactions of Thr·PNHE were observed over a serum pH range of 5.4 to 7.4 in all six patients studied. Maximal hemolysis in the thrombin test system varied from 25% to 90% in these patients.

Antiglobulin neutralization tests. The ability of antiwhole serum, neutralized by γ-globulin, β_{1E}-globulin, β_{1C}-globulin, and β_{1C}-globulin plus β_{1E}-globulin, to agglutinate AH·PNHE and Thr·PNHE was studied in five patients. The agglutination of AH·PNHE by antiwhole serum was not affected by prior incubation of the antiserum with γ-globulin or β_{1E}-globulin alone (Table V). Beta$_{1C}$-globulin alone and β_{1C}-globulin plus β_{1E}-globulin, however, completely inhibited the reaction. The agglutination of Thr·PNHE by anti-

TABLE V

Agglutination of AH·PNHE by antiwhole serum neutralized with various proteins

Neutralizing protein	Patient no. 1	2	4	5	7
	Agglutination scores*				
None	2	1½	2	1½	2
γ-Globulin	2	1½	2	1½	2
β_{1E}	2	1½	2	1½	2
β_{1C}	0	0	0	0	0
$\beta_{1C} + \beta_{1E}$	0	0	0	0	0

* 4, maximal agglutination; 0, no agglutination; other, intermediate agglutination.

whole serum likewise was not inhibited by prior incubation of the antiserum with γ-globulin or β_{1E}-globulin alone (Table VI). $Beta_{1C}$-globulin alone produced partial inhibition. $Beta_{1C}$-globulin plus β_{1E}-globulin completely inhibited the reaction.

The results of these neutralization studies confirm the presence of β_{1C}-globulin on AH·PNHE and of β_{1C}-globulin plus β_{1E}-globulin on Thr·PNHE. In addition they would suggest that these are the only human proteins detectable on these cells by the methods employed. However, the preparations of β_{1C}-globulin (12) used in these neutralization experiments and in preparing the anti-β_{1C}-globulin have recently been shown to contain trace amounts of two other previously unrecognized complement proteins (17). These appear to follow directly β_{1C}-globulin in the complement sequence. The presence of these substances on AH·PNHE and Thr·PNHE cannot be excluded at present, although evidence to date suggests that these proteins are not firmly fixed to cell-complement complexes as is the case with β_{1C}-globulin (17).

TABLE VI

Agglutination of Thr·PNHE by antiwhole serum neutralized with various proteins

Neutralizing protein	Patient no. 1	2	4	5	7
	Agglutination score*				
None	3½	2	3	2½	3
γ-Globulin	3½	2	3	2½	3
β_{1E}	3½	2	3	2½	3
β_{1C}	2	1	1	1	1½
$\beta_{1C} + \beta_{1E}$	0	0	0	0	0

* 4, maximal agglutination; 0, no agglutination; others, intermediate agglutination.

Discussion

The relationship of the complement system to serum factors required for *in vitro* PNH acid hemolysis has been the subject of considerable investigation. The many discrepancies between the acid hemolysis reaction and the usual complement-dependent hemolytic systems have been recently summarized (3). Nonetheless, Ham and Dingle (18) and Hinz, Jordan, and Pillemer (19) have demonstrated that serum treated so as to remove individual complement components fails to support PNH acid hemolysis. The present demonstration of complement components on the residual unlysed PNH erythrocytes from the acid hemolysis and thrombin test systems provides even more direct evidence for the participation of complement in these two hemolytic reactions.

In interpreting the data presented in this paper, it is useful to compare and contrast the details of complement coating in EAC′ systems and in the two PNH hemolytic systems under study. In EAC′ hemolysis an orderly series of events takes place on the erythrocyte surface beginning with the attachment of antibody and proceeding with fixation of the components of complement in the sequence $EAC'_{1,4,2,3}$ (*7b*, *20a*). In reactions involving human complement, β_{1E}-globulin has been identified as C′4 and β_{1C}-globulin as the first reacting of the several subcomponents of classical C′3 (*20b*). Under favorable conditions the above reaction goes to completion, and hemolysis of the erythrocyte ensues, presumably due to complement-mediated membrane damage (21, 22). Both β_{1C}-globulin and β_{1E}-globulin are detected on residual, unlysed, normal erythrocytes from human EAC′ systems, using the antiglobulin agglutination technique (5, 6). By neutralization studies β_{1C} and β_{1E} appear to be the predominant complement components on such cells (5, 23) possibly because of their greater antigenicity or their more permanent fixation to these cells.

In this respect normal erythrocytes surviving EAC′ hemolysis resemble PNH erythrocytes surviving thrombin test hemolysis, which are similarly coated with β_{1C}- and β_{1E}-globulins. These results are not surprising, since commercial thrombin is known to contain agglutinating antibodies (24) that have been shown to fix complement to normal erythrocytes and PNHE as well as to en-

hance the PNH acid hemolysis reaction (25). Whether these antibodies account entirely for the ability of bovine thrombin to enhance PNH acid hemolysis remains to be determined (3).

In contrast, erythrocytes surviving acid hemolysis are coated with β_{1C}-globulin but not β_{1E}-globulin. The apparent lack of antibody in the acid hemolysis reaction (18) implies that hemolysis of PNHE in this system occurs by direct attachment of complement to the PNHE during the reaction. The absence of β_{1E} (C'4) and presence of β_{1C} (C'3 subcomponent) on the AH·PNHE suggest that this direct attachment of complement involves a component acting after C'4 and before or at the β_{1C} step. Accordingly, this attachment must occur at either the C'2 or β_{1C} step, since these are the next two components to act after C'4 (*20a,b*). Yachnin's demonstration that hemolysis of PNHE can be supported by isolated subcomponents of C'3 (26) favors direct complement attachment occurring at the β_{1C} step rather than the C'2 step.

The above considerations suggest that the defect of the PNH erythrocyte predisposing to hemolysis involves a heightened vulnerability to the action of C'3 components. Other recent observations are consistent with this formulation. Yachnin and Ruthenberg (15) have shown that when human complement is used in EAC' systems, EAC' hemolysis, like PNH acid hemolysis, proceeds maximally at pH 6.5. The increase in EAC' hemolysis occurring at this pH appears to be due to greater efficiency of the C'3 step. Likewise Rosse and Dacie (27) have presented evidence that the increased sensitivity of PNHE to hemolytic antibodies appears to be due to increased efficiency in the later steps of the complement reaction in systems employing PNHE.

If the heightened sensitivity of PNHE to hemolysis relates to its greater susceptibility to C'3 damage, and if acid hemolysis does proceed by direct attachment of a later-acting component such as β_{1C}-globulin, the question then arises as to how such events may relate to the kinetics of the acid hemolysis reaction. Yachnin and Ruthenberg have recently presented evidence to suggest that PNH acid hemolysis may be a two phase reaction (28). In the initial or serum phase there is generation of hemolytically active, late-acting components which then attach directly to the abnormal PNH erythrocyte to bring about hemolysis. The demonstration of β_{1C}-globulin but not β_{1E}-globulin on the AH·PNHE is quite consistent with this hypothesis.

Such a view implies that the important determinants of PNH acid hemolysis (pH, temperature) operate independently to activate a late-acting component such as β_{1C}-globulin regardless of whether PNH erythrocytes are present in the system. The PNH erythrocyte, being defective, is subject to injury in this setting, whereas the normal erythrocyte is not. This interpretation would account for the seeming paradox that no more complement is utilized in an acid hemolysis system employing PNHE than in one employing normal erythrocytes (18) despite the fact that striking hemolysis of PNHE occurs.

The relationship of the findings of the present study to *in vivo* PNH hemolysis remains to be determined. The direct antiglobulin test is usually negative in PNH (29). However, there have been sufficient reports of positive direct antiglobulin reactions in PNH patients to suggest that the incidence of this phenomenon is greater than that due to chance alone (30). Whether these instances represent the superimposition of autoantibodies in PNH or intervals when complement, having attached directly to the PNHE *in vivo,* can be detected remains to be determined. Further studies of such patients utilizing specific antisera to γ-globulin and complement components may help clarify this question.

Summary

Paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes surviving hemolysis in the *in vitro* acid hemolysis and thrombin test systems were studied and found to be coated with complement. PNH erythrocytes surviving thrombin test hemolysis were coated with both β_{1C}-globulin and β_{1E}-globulin, whereas only β_{1C}-globulin could be detected on the surface of PNH erythrocytes surviving acid hemolysis. These results provide more direct evidence than previously available for the participation of complement in these two reactions.

PNH erythrocytes surviving thrombin test hemolysis resemble normal cells surviving hemolysis in human erythrocyte-antibody-complement systems in that they are coated with both β_{1C}-globulin and β_{1E}-globulin. This suggests a role of com-

plement-fixing antibodies known to be present in commercial bovine thrombin preparations. PNH erythrocytes surviving acid hemolysis, in contrast, are coated with β_{1C}-globulin but not β_{1E}-globulin. These results are consistent with a hypothesis of direct interaction of subcomponents of the third complement component and the intrinsically defective PNH erythrocyte to account for the increased sensitivity of this cell to complement damage.

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