LYSIS OF ERYTHROCYTES BY COMPLEMENT IN THE ABSENCE OF ANTIBODY* ‡

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In classical immune cytolysis, antibody bound to cell-surface antigen initiates the lytic action of complement. It permits binding and activation of the first component of complement which in turn catalyzes binding to the cell surface of the second and fourth components, setting in motion a well defined chain of events which results in functional membrane impairment (1).

In this paper alternative mechanisms of cell lysis by complement will be described which are entirely independent of antibody. One mechanism of lysis of the nonsensitized target cell is initiated by the $\overline{C4,2}$ enzyme[1] either from the fluid phase or from the surface of another cell. Action of the enzyme on subsequent complement proteins affects the "by-standing" target cell in a characteristic manner. Beginning with the fifth component, activated complement proteins become physically bound to the target cell, which after reaction with C8 and C9, undergoes lysis. Whereas this mechanism is relatively inefficient when compared with classical immune cytolysis, it points out the possibility of a lytic effect occurring at some distance from the site of activation of the initially acting complement components. Erythrocytes (E) from patients with paroxysmal nocturnal hemoglobinuria (PNH)[2] were found to be particularly susceptible to lysis induced by fluid phase $\overline{C4,2}$.

Another mechanism of nonimmune lysis involves only the six late-acting complement proteins and the C3 serum proinactivator (2). After its activation

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[1] Terminology employed conforms with the recommendations of the World Health Organization Committee on Complement Nomenclature (*Bull. World Health Organ.* 1968. **39**:939).

[2] *Abbreviations used in this paper:* E, washed erythrocytes; EA, washed, optimally sensitized sheep erythrocytes; GVB, isotonic NaCl-veronal buffer containing 0.1% gelatin, 0.00015 M calcium, and 0.0005 M magnesium; GVBE, isotonic NaCl-veronal buffer containing 0.1% gelatin and 0.02 M EDTA; PNHE, washed erythrocytes from patients with paroxysmal nocturnal hemoglobinuria.

in whole sheep or guinea pig blood, complement-dependent hemolysis has been reported to occur (3, 4). It will be shown that, unlike normal human erythrocytes, PNH cells are exceptionally susceptible to this mode of hemolysis.

The results contained in this paper have been presented in part in the form of two abstracts (5, 6).

Materials and Methods

Purified Complement Proteins and Complement Reagents.—Partially purified, macromolecular C1 was prepared from human serum according to Nelson (7) and C1s was isolated using either of the two published methods (8, 9). C2 was purified as described previously (10) and was used exclusively in its oxidized form (11). C3 (12), C4 (13), C5 (12), C8 (14), and C9 (15) were obtained according to published methods.

Isolated C3 and C5 were utilized for quantitative uptake studies in radioactively labeled form. The chloramine T method (16) was used and the average specific radioactivity was, respectively, 50,000 and 120,000 cpm/μg of C3 and C5.

Partially purified C6 was prepared as follows. The euglobulin fraction was precipitated from serum by dialysis against a 0.008 M EDTA solution of pH 5.4 and a conductance at 22°C of 1.25 mmho/cm. The protein precipitate was dissolved in 0.02 M sodium phosphate buffer, pH 7.3, containing approximately 0.2 M sodium chloride. After clearing the solution by centrifugation for 60 min at 36,000 rpm in a Spinco No. 40 rotor (Beckman Instrument Co., Spinco Division, Palo Alto, Calif.), it was dialyzed against 0.02 M sodium phosphate buffer, pH 7.3, overnight and applied to a 3.3 × 70 cm column of triethylaminoethyl (TEAE) cellulose equilibrated with buffer used for dialysis. The chromatogram was developed with a combined salt and pH gradient, the limiting solution being 0.3 M NaH$_2$PO$_4$. C6 was eluted between pH 6.5 and 6.0, corresponding to a conductance range of 6.0–7.5 mmho/cm. C6 activity was detected using washed, optimally sensitized sheep erythrocytes (EA) and C6-deficient rabbit serum (17). The fractions containing the greatest activity were pooled and concentrated by pressure filtration in an Amicon apparatus (Amicon Corp., Lexington, Mass.) using a UM 10 membrane. The material was then further separated by chromatography on a 2.5 × 38 cm column of carboxymethyl (CM) Sephadex C-50 equilibrated with sodium phosphate buffer, pH 6.0, ionic strength 0.05, containing sufficient NaCl to yield a conductance of 10 mmho/cm. A NaCl gradient was employed, the limiting conductance being 50 mmho/cm. C6 activity was eluted at a conductance range of 14.5–17.5 mmho/cm. This preparation was concentrated, frozen in liquid nitrogen, and stored at −70°C and used throughout this study. Approximately 60 ng of the preparation gave 50% lysis of 5 × 10^7 EA in 60 min at 37°C (total volume 1.5 ml), in presence of 6.6% of C6-deficient rabbit serum.

Partially purified C7 was prepared from the pseudoglobulin fraction of human serum, i.e., the supernatant of the euglobulin preparation (see above). The pH of the protein solution (400 ml) was adjusted to 7.3 with 1 NNaOH and the conductance was raised to 2.2 mmho/cm with NaCl. The material was applied to a 7.5 × 50 cm column of TEAE cellulose equilibrated with 0.02 M sodium phosphate buffer, pH 7.3, and was eluted stepwise with the same buffer containing NaCl to yield 10 mmho/cm. The activity emerged within a conductance range of 4.0–6.5 mmho/cm. C7 activity was assayed with EAC1-3 plus an excess of C5, 6, 8, and 9. The active material was concentrated to 15 ml and applied to a 5 × 120 cm column of Sephadex G-200 equilibrated with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 8.0, containing 0.50 M NaCl. C7 activity was detected in fractions between the second and third peak of the elution profile. The active protein was then applied to a 2 × 30 cm column of P-cellulose (Cellex P, Bio-Rad Laboratories, Richmond, Calif.) equilibrated with sodium phosphate buffer, pH 6.0, ionic strength 0.1. After elution by NaCl concentration

gradient, C7 activity was found in fractions of 17–23 mmho/cm. With the assay system used, approximately 280 ng of C7 gave 50% lysis of 5×10^7 cells in 60 min at 37°C.

In some experiments a euglobulin subfraction was used as a source for C3, 5, 6, 7, and 8 (C3-8 reagent). This was obtained as described (15). A reagent supplying C6-9 was prepared by treating guinea pig serum with hydrazine and KCNS (18) and diluting it in isotonic NaCl-veronal buffer containing 0.1% gelatin and 0.02 M EDTA (GVBE) of pH 7.4.

Cobra Factor.—The cobra factor was isolated from lyophilized Naja naja venom purchased from Ross Allen Reptile Farm, Silver Springs, Fla. The method of isolation has previously been described (19).

Cells.—Sheep erythrocytes were purchased from the Colorado Serum Company, Denver, Colo. Normal human erythrocytes were obtained from laboratory personnel. Erythrocytes from four different patients with PNH were kindly supplied by Dr. Lawrence Petz, Harkness Community Hospital, San Francisco, Calif. and Doctors Robert Hartmann and David Jenkins, Nashville, Tenn.

Conditions of Lysis of Nonsensitized Erythrocytes.—In experiments where lysis was induced from the fluid phase, 2×10^8 washed erythrocytes (sheep E) or 1×10^8 human E were packed by centrifugation into the bottom of a small test tube and the supernatant was removed with a Pasteur pipette. Usually C3, 5, 6, and 7 were then added and the cells suspended by use of a Vortex mixer. After addition of $\overline{C4,2}$ the reaction mixture was incubated for 20 min at 37°C. The diluent was GVBE, pH 6.5. The total reaction volume was 0.25–0.3 ml. The cells were washed twice in GVBE of pH 7.4 and suspended in 2 ml of this buffer. 0.5 ml of the suspension was reacted with 1 ml of a solution of C8 and C9 for 60 min at 37°C. Controls included 2×0.5 ml of cell suspension plus 1 ml buffer and 1 ml distilled water, respectively. The free hemoglobin concentration was measured spectrophotometrically at 412 nm.

The $\overline{C4,2}$ enzyme was prepared by mixing, in a total of 1 ml, 12×10^{10} effective molecules of $^{\mathrm{oxy}}C2$ (approximately 7 μg protein), 3×10^{10} effective molecules of C4 (approximately 3 μg protein), and 0.75 μg of C1s. The pH was adjusted to 7.4 and the Mg ion concentration to 0.0005 M. This mixture was incubated at 37°C for 30 min after which time EDTA was added to a final concentration of 0.02 M, and the pH was lowered to 6.5 with 0.1 N HCl. The final volume was 1.5 ml, of which 0.05–0.1 ml were used per 2×10^8 sheep E or 1×10^8 human E, unless otherwise indicated.

The amount of C3 used for the same number of cells was usually 20 μg and was varied in some experiments between 5 and 100 μg. The amount of C5 was generally 15 μg per tube and was varied in dose response experiments over a 10,000-fold range. C6 and C7 were employed in optimal concentrations, which cannot be defined by weight since only functionally pure material was available. C8 and C9 were used in amounts of 26 and 60 ng per tube, respectively.

In experiments where target-cell lysis was induced by trigger cells, $\overline{C4,2}$ or $\overline{C4,2,3}$ were bound to the trigger cells according to published methods (20). The number of $\overline{C4,2}$ or $\overline{C4,2,3}$ complexes per cell was 3000–5000. Usually 1×10^8 ^{51}Cr-labeled sheep E was mixed with 1×10^8 trigger cells and a cell button was formed by centrifugation. The supernatant was withdrawn and replaced by 0.2 ml of a solution of C3, 5, 6, 7 or C5, 6, 7 in pH 6.5 buffer. The mixture was incubated for 20 min at 37°C, washed twice in 4 ml pH 7.4 buffer, resuspended in 2 ml of the latter buffer and 3×0.5 ml of this suspension were treated and evaluated as described above for lysis induced from the fluid phase. ^{51}Cr release was measured using 1 ml of the cell-free supernatant obtained after completion of the reaction.

In experiments where lysis of human erythrocytes was induced by cobra factor, 5×10^7 red cells were incubated with 0.1 ml of a blood group-compatible human serum in the presence of purified cobra factor in a total reaction volume of 0.310 ml. The pH had been ad-

justed to 8.0 and the Mg ion concentration to 0.0005 M. After 30 min at 37°C, 2 ml of iso-
tonic NaCl-veronal buffer containing 0.1% gelatin, 0.00015 M calcium, and 0.0005 M mag-
nesium (GVB) of pH 8.0 was added to each tube and incubation was continued for another
60 min.

Labeling of E with ^{51}Cr.—10 ml of sheep E containing 5×10^8 cells were incubated with
0.25–0.5 mCi of ^{51}Cr sodium chromate (New England Nuclear Corp., Boston, Mass.) for
45 min at 37°C in GVBE of pH 6.5. The cells were washed with 5×10 ml of the same buf-
fer and resuspended to obtain the desired concentration. The uptake of label corresponded
to 20,000–40,000 cpm per 2×10^8 cells.

Serological Detection of Cell-Bound Complement Proteins.—Cell-bound C3, C4, or C5 were
detected using monospecific complement antisera and the microtiter technique (Cooke En-
gineering Co., Alexandria, Va.). 0.025 ml samples of 5×10^7 cells/ml were mixed with dilu-
tions of the antisera ranging from 1:50 to 1:6400. The plates were kept for 20 min at 37°C
and overnight at 4°C, and the reaction was evaluated by reading the settling patterns.

Cell-bound C3 was also detected by immune adherence (21, 22) using lysates prepared
from cell suspensions containing 5×10^6 to 1×10^9 cells/ml.

RESULTS

Complement-Dependent Lysis of Nonsensitized Sheep Erythrocytes Initiated by C̄4,2 in Free Solution

Requirement of Components.—Treatment of nonsensitized sheep erythrocytes
(E) with C3,5,6,7 in presence of preformed C̄4,2 rendered these cells sus-
ceptible to lysis by C8 and C9. Similarly, incubation of E with C3-8 in pres-
ence of C̄4,2 caused these cells to become susceptible to the action of C9.
Since treatment of E was performed in two consecutive steps which were
separated by repeated washings of the cells, it is evident that the first treat-
ment led to the formation of intermediate complexes carrying either C7 or C8
sites.

Deletion from the reaction mixture of either the activating enzyme C̄4,2 or
the components C3-7 or C3-8 prevented lysis. Also, when E was first incubated
with C̄4,2 plus C3 and then washed, these cells were unable to undergo lysis
upon later addition of C5-9. The latter control indicated that fluid phase
C̄4,2 did not form hemolytically active C3 sites on the surface of E. The results
are summarized in Table I.

Relationship Between Lysis of E and the Concentration of C̄4,2 and C5.—
Fig. 1 shows that lysis of E is proportional to the concentration of the C̄4,2
enzyme in the reaction mixture. In each of the two experiments depicted, the
amounts of the other complement proteins were constant. When the concen-
tration of C5 was varied and that of all other proteins including C̄4,2 was kept
constant, lysis of E was found to be proportional to C5 input (Fig. 2).

Physical Uptake of Complement Proteins by E.—Since E treated with C3,
5,6,7 and C̄4,2 acquired reactivity with C8 and C9, the question arose as to
which complement proteins were specifically bound to their surface. Qualita-
tive detection was attempted using antisera to three different complement

proteins. In all of five separate experiments, treated E failed to react with anti-C3 and anti-C4, but gave a definite agglutination reaction with anti-C5. The absence of specifically bound C3 was further substantiated by the observation of negative immune adherence reactivity (Table II). On the other hand,

TABLE I

Lysis of Nonsensitized Erythrocytes (E) by Complement Following Activation in Free Solution

Treatment of E			Hemolysis
I	II	III	
			%
C3-8 + C$\overline{4,2}$	—	C9	89.4
C3-8	—	C9	1.8
C3,5,6,7 + C$\overline{4,2}$	—	C8,9	56.1
C3,5,6,7	—	C8,9	3.9
C$\overline{4,2}$	—	C8,9	0
C$\overline{4,2}$	—	C-EDTA	2.2
C3 + C$\overline{4,2}$	C5,6,7	C8,9	2.9

Conditions: Treatment I: 2×10^8 E, reagents in 0.2 ml GVBE, pH 6.5, 20 min at 37°C; the cells were then washed twice. Treatment II: conditions as in I. Treatment III: 5×10^7 E, reaction volume 1.5 ml, 60 min at 37°C.
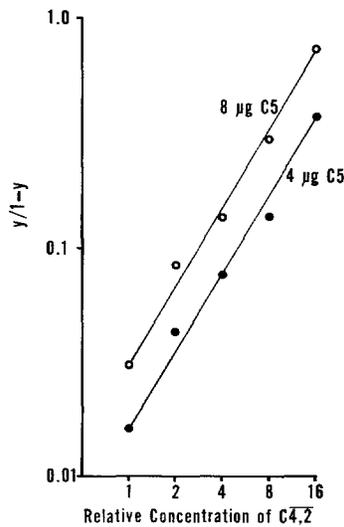

Fig. 1. Dependence of lysis of nonsensitized sheep erythrocytes upon the concentration of the C$\overline{4,2}$ enzyme in the reaction mixture. Two different amounts of C5 were chosen; the C$\overline{4,2}$ concentration was varied over a 16-fold range, the concentration of all other complement proteins was constant. $y/1 - y = 1$ corresponds to 50% lysis.

EAC$\overline{4,2,3}$ and EAC$\overline{4,2,3}$,5 gave strongly positive serological reactions as anticipated. These findings indicated that the treated E cells which could be lysed with C8 and C9, but lacked C3 and C4, were in the state EC5,6,7.

The serological data were confirmed and extended by quantitative uptake studies using radiolabeled C3 and C5. Table III lists results of experiments with ^{125}I-C3 in which the input of C3 was varied and in which uptake was determined in the presence and absence of C$\overline{4,2}$ or C5,6,7. Compared to EAC$\overline{4,2}$, which showed a C3 uptake of 15% of input or more than 14,000 molecules per
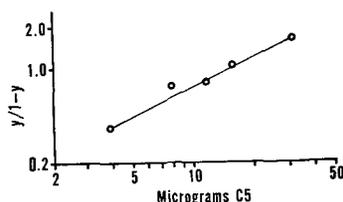

Fig. 2. Dependence of lysis of nonsensitized sheep erythrocytes upon C5 concentration. C5 input was varied from 4 to 35 μg. All other complement components were constant; the concentration of the C$\overline{4,2}$ enzyme corresponded to 16 of Fig. 1.

TABLE II

Serological Analysis of Nonsensitized Erythrocytes (E) after Reaction with Activated Complement Components

Treatment	Agglutination by antiserum to			Immune adherence
	C3	C4	C5	
E + C3,5,6,7 + C$\overline{4,2}$	0	0	+	0
E + C3,5,6,7	0	0	0	0
EAC$\overline{4,2,3}$	+	+	0	+
EAC$\overline{4,2}$,3,5	+	+	+	+

cell, the nonsensitized E exhibited an uptake of approximately 1%. Controls lacking the C$\overline{4,2}$ enzyme or EAC1,4 cells treated with C3 similarly took up 1% of the amount of C3 offered. Thus, there was no detectable significant specific C3 uptake by E. Furthermore, E, having bound a total of 1080 C3 molecules per cell, were entirely negative in the anti-C3 agglutination reaction. This test detects as few as 100 specifically bound C3 molecules per cell.

In the case of C5, uptake by EAC$\overline{4,2,3}$ was 1.9% of input, in agreement with previous studies (18). Nonspecific adsorption to EAC$\overline{4,2}$ was 0.95%. The specific uptake therefore may be calculated to be 1% or 1480 molecules per cell. Similarly, the specific uptake of C5 by nonsensitized E was approximately 1% of input, corresponding in two experiments respectively to 600 and 438

C5 molecules per cell. This degree of C5 binding must be considered highly significant (Table IV). In addition, these cells could be agglutinated with an anti-C5 serum as indicated in Table II. The control cells with nonspecifically adsorbed C5 on their surface gave no agglutination reaction.

TABLE III

Uptake of ^{125}I-*C3 by Nonsensitized Erythrocytes* (E) *Following Activation in Free Solution*

| ^{125}I—C3 input | Reaction mixture | Uptake | | Specific uptake* Molecules/cell | Lysis |
		Input	Molecules/ cell		
(μg)		(%)			(%)
5.5	E + C3,5,6,7 + C$\overline{4,2}$	1.31	1,080	478	58.0
5.5	E + C3,5,6,7	0.73	602		4.3
18	E + C3,5,6,7 + C$\overline{4,2}$	0.73	1,970	−1,189	54.8
18	E + C3,5,6,7	1.17	3,159		5.8
6.4	E + C3 + C$\overline{4,2}$	1.38	1,324	614	ND‡
6.4	E + C3	0.74	710		ND
18	E + C3 + C$\overline{4,2}$	0.20	540	−480	ND
18	E + C3	0.38	1,020		ND
6.4	EAC$\overline{4,2}$ + C3	15.41	14,793	13,670	ND
6.4	EAC4 + C3	1.17	1,123		ND
18	E + C3i	1.16	3,130		

Conditions: 2 × 10⁸ cells, reagents in 0.2 ml GVBE, pH 6.5, 20 min, 37°C. The cells were then washed five times and uptake of ^{125}I was measured. To determine the degree of hemolysis, 5 × 10⁷ treated cells were incubated with C8 and C9 in a total volume of 1.5 ml for 60 min at 37°C.

* Difference between uptake in presence and absence of C$\overline{4,2}$.

‡ Not determined.

Complement-Dependent Lysis of Nonsensitized Sheep Erythrocytes Triggered by EAC$\overline{4,2}$ or EAC4,2,3

Requirement of Components.—The results obtained so far raised the question whether the activating enzyme may be bound to one cell and yet mediate up_take of C5,6,7 by another cell. Therefore, EAC$\overline{4,2,3}$ was mixed with ⁵¹Cr-labeled E and the cell mixture was treated first with C5,6,7, washed, and then with C8 and C9. As shown in Table V, ⁵¹Cr was released under these conditions, indicating lysis of 25% of E present. In addition, there was complete lysis of the trigger cells, EAC$\overline{4,2,3}$. There was no chromium release when C3 was omitted from the reaction mixture. This and similar experiments showed that

C5,6,7, when activated by cell-bound $\overline{C4,2,3}$, may transfer and become bound to an adjacent target cell, thereby initiating the cytolytic reaction.

To determine whether a hemolytically reactive EC5 intermediate can be prepared the following experiment was carried out. $EAC\overline{4,2,3}$ was mixed witn ^{51}Cr E and the mixture was treated for 10 min at 30°C with various amounts of C5. The cells were then washed and reacted with a reagent containing C6-9.

TABLE IV

Uptake of ^{125}I-C5 by Nonsensitized Erythrocytes (E) Following Activation in Free Solution

^{125}I-C5 input	Reaction mixture	Uptake		Specific uptake* Molecules/cell	Lysis
		Input	Molecules/ cell		
(μg)		*(%)*			*(%)*
4	E + C3,5,6,7 + $C\overline{4,2}$	2.51	1,506	438	13.3
4	E + C3,5,6,7	1.78	1,068		0.2
4	E + C3,5,6,7 + $C\overline{4,2}$	2.65	1,590	600	35.4
4	E + C3,5,6,7	1.65	990		6.6
10	EAC4 + C5,6,7	0.90	1,350		ND‡
10	$EAC\overline{4,2}$ + C5,6,7	0.95	1,420		ND
10	$EAC\overline{4,2,3}$ + C5,6,7	1.93	2,900	1,480	ND

Conditions: See Table III.
* Difference between uptake in presence and absence of $C\overline{4,2}$.
‡ Not determined.

TABLE V

Lysis of Nonsensitized Erythrocytes (E) by C5-C9 after Activation by $EAC\overline{4,2,3}$

Reaction mixture	Lysis of E		Lysis of EAC	
	No.	*%*	*No.*	*%*
E + $EAC\overline{4,2,3}$ + C5,6,7	2×10^7	25	2×10^7	100
E + $EAC\overline{4,2}$ + C5,6,7	0	0	0	0

Conditions: 2×10^7 EAC, 8×10^7 E; reagents in 0.2 ml GVBE, pH 6.5; incubation, 30 min at 37°C, followed by washing and exposure to C8,9 for 60 min at 37°C. E labeled with ^{51}Cr; lysis of E measured by ^{51}Cr release.

The amount of chromium released appeared insignificant and bore no relationship to the increasing amounts of C5 added (Table VI). It was concluded that C5 cannot be bound to E specifically and in hemolytically active form without C6 and C7.

Dependence of Lysis of E Upon Cell Concentration.—Fig. 3 demonstrates that lysis of nonsensitized E by $EAC\overline{4,2}$ and C3-9 is markedly dependent on a high cell concentration. Lysis of E was 44% at the highest concentration tested

TABLE VI

Failure of Activated C5 to Bind in Hemolytically Active Form to E in Absence of C6 and C7

C5 input	Treatment after reaction with C5	Lysis of	
		E	EAC$\overline{4,2,3}$
(µg)		(%)	(%)
2.5	C6-9	2.6	100
5	C6-9	2.6	100
10	C6-9	2.8	100
20	C6-9	3.3	100
2.5	C8,9	0.2	1.7
5	C8,9	0.2	1.2
10	C8,9	0.2	1.7
20	C8,9	0.2	1.9
—	C6-9	0.2	11.5
—	C8,9	0.2	2.6

Conditions: 10^8 E and 10^8 EAC$\overline{4,2,3}$ in GVBE, pH 6.5, were treated with C5 for 10 min at 30°C in a total volume of 0.3 ml. The cells were washed twice, resuspended with 0.2 ml C6-9 or C8 and C9 and incubated for 20 min at 37°C. To every tube was then added 2.0 ml of GVBE, pH 7.4, and incubation was continued for another 60 min. E was labeled with ^{51}Cr and lysis of E was detected by ^{51}Cr release.
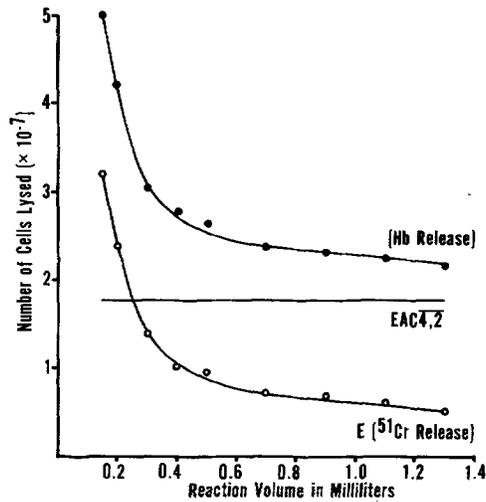

FIG. 3. Dependence of lysis of nonsensitized sheep erythrocytes upon the cell concentration. 2×10^7 EAC$\overline{4,2}$ and 8×10^7 ^{51}Cr-labeled E were incubated together in increasing volumes of the same dilution of C3-C8. After 20 min at 37°C the cells were washed and lysed with C9. The upper curve (Hb release) shows the total number of cells lysed; the lower curve (^{51}Cr release) shows the number of E lysed. The horizontal line indicates the (calculated) number of EAC$\overline{4,2}$ lysed.

(6.6 × 10⁸ cells/ml) and sharply decreased to less than 10% at 2.8 × 10⁸ cells/ml.

Comparative Efficiency of C5 in the Lysis of E and EAC4,2,3.—Equal numbers of ⁵¹Cr E and EAC4,2,3 were mixed and were reacted at a cell concen-
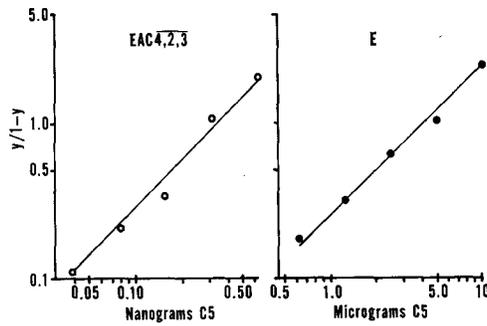

FIG. 4. Comparative efficiency of C5 in lysing E and EAC4,2,3. Equal numbers of ⁵¹Cr-labeled E and EAC4,2,3 were reacted with C5-C9 in the same reaction mixture. The C5 input was varied between 0.04 ng and 10 μg. The concentration of all other components was constant. Lysis of EAC4,2,3 was calculated from the total hemoglobin release and the release of ⁵¹Cr.
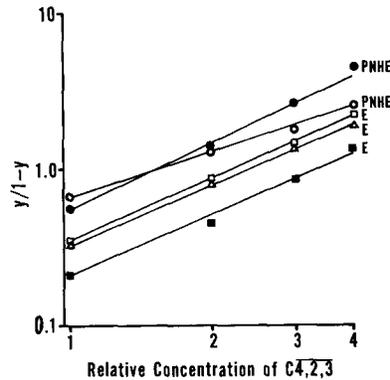

FIG. 5. Dependence of lysis of nonsensitized PNH and normal human erythrocytes upon the concentration of C4,2,3 in the reaction mixture. The C4,2,3 concentration was varied over a fourfold range, whereas the concentration of the other complement components was constant. Erythrocytes from two patients with PNH and from three normal individuals were used.

tration of 6.6 × 10⁸ cells/ml with C5-9. Whereas all other parameters were constant, the C5 concentration was varied from 0.04 ng to 10 μg. As shown in Fig. 4, 50% lysis of EAC4,2,3 was obtained with 0.34 ng of C5, whereas the same degree of lysis of E required 4.1 μg of C5.

Complement-Dependent Lysis of PNH and Normal Human Erythrocytes Initiated in Free Solution

Relationship Between Lysis and the Concentration of $\overline{C4,2,3}$ and C5.—Fig. 5 shows the extent of lysis of PNH and normal human erythrocytes as a function of $\overline{C4,2,3}$ concentration. 50% lysis of PNH cells was observed with a relative amount of $\overline{C4,2,3}$ of 1.5 and the same degree of lysis of normal cells was ob-
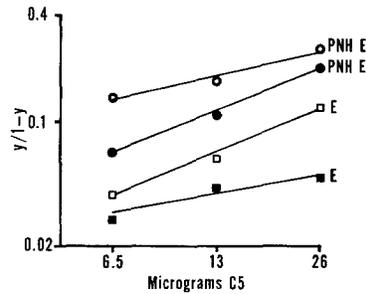

FIG. 6. Dependence of lysis of nonsensitized PNH and normal human erythrocytes upon the C5 concentration.
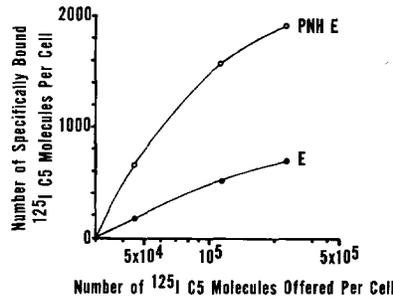

FIG. 7. Comparison of the binding efficiency of activated C5 by PNH and normal human erythrocytes. PNHE and E were each incubated with varying amounts of ^{125}I-C5 and constant amounts of $\overline{C4,2}$, C3, C6, and C7. After 20 min at 37°C, the cells were washed and the uptake of radiolabel was determined.

tained within a relative amount of 2.2–3.4. Lysis of these two types of cells as a function of C5 concentration is demonstrated in Fig. 6. To achieve the same degree of lysis, 3.5–11 μg of C5 were required for PNH cells and 23–100 μg of C5 for normal cells.

Relationship Between C5 Uptake and Cell Lysis.—Under identical conditions and in the presence of fluid phase $\overline{C4,2,3}$ PNH erythrocytes bound more than three times the number of C5 molecules than normal human erythrocytes (Fig. 7). The degree of ensuing hemolysis was proportional to the number of C5

molecules bound. When the same number of C5 molecules had become bound to either cell type, the same degree of hemolysis was observed (Fig. 8).

Complement-Dependent Lysis of PNH Erythrocytes Triggered by Cobra Factor. —Isolated cobra factor was used to activate the C3 serum proinactivator.
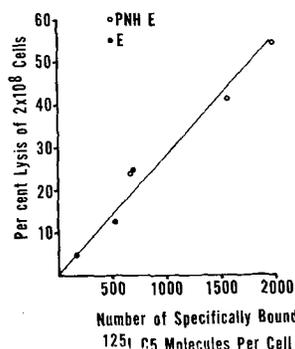

FIG. 8. Comparison of the hemolytic efficiency of C5 bound to PNH and to normal human erythrocytes. Same experiment as Fig. 7. Lysis was determined after incubation of the washed cells with C8 and C9.
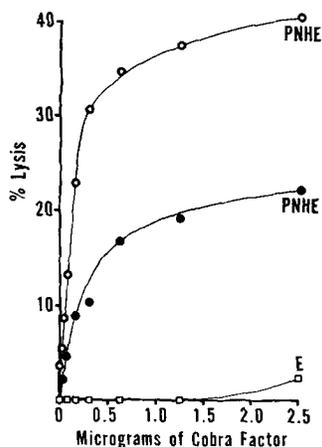

FIG. 9. Comparison of lysis of PNH and normal human erythrocytes induced by cobra factor. 5×10^7 cells were incubated with 0.1 ml of blood group-compatible serum and varying amounts of cobra factor in a total volume of 0.310 ml. The diluent was GVB, pH 8.0. After 30 min at 37°C, 2.0 ml of GVB of pH 8.0 were added to each tube and incubation was continued for another 60 min.

Addition of increasing amounts of cobra factor to samples of human serum containing PNH cells and incubation at 37°C and pH 8.0 led to development of marked hemolysis (Fig. 9). Subsequent examination of lysed and unlysed cells with antisera to complement proteins gave positive agglutination reactions. Normal human erythrocytes were unaffected by this mechanism of hemolysis.

DISCUSSION

The present experiments define a new pathway of complement-mediated hemolysis which is not dependent on the participation of antibody and on the presence of the first four complement components at the surface of the target cells. The attack of complement proteins on the target cell membrane begins with C5 and involves only the five terminal components. This study represents a detailed exploration of preliminary observations which were made several years ago (23). The mechanism described resembles, to a certain extent, that postulated by Yachnin for PNH-cell lysis (24) and may be related to the mechanism underlying the phenomenon of reactive hemolysis reported by Thompson and Lachmann (25). The results demonstrate that activated C5 in the presence of C6 and C7 may bind directly and in hemolytically active form to receptors of unsensitized erythrocytes. They show that C5, C6, and C7 act as a functional unit in this reaction and that the three proteins can transfer from the site of activation on one cell to the site of binding on another cell.

The nature of the binding site of C5 in classical immune hemolysis is still unknown, although it has been clearly shown that firm, physical binding of C5 is a prerequisite of the formation of hemolytically active C5 sites on the cell surface (26–28). Shin et al. (28) believe guinea pig C5 to be bound in close proximity to the C2 and C3 molecules on $EAC\overline{4,2,3},5$. This assumption is based on their observation that C5 is dissociated from cells concomitant with the decay-release of C2. Release of specifically bound ^{125}I-C5 could not be demonstrated for the human analog, neither in conjunction with C2 release nor as a consequence of decay of cell-bound hemolytic C5-sites (18). Thus, the hypothesis that the receptor of C5 is the $C\overline{4,2,3}$ complex can neither be verified nor refuted on the basis of this study. However, the reported formation of EC5,6,7 cells shows that C5,6,7 sites may be established directly on membrane receptors without the previous binding to this membrane of $C\overline{4,2,3}$ or of antibody.

Specific binding of C5 by E required the participation of C6 and C7, since a hemolytically reactive EC5 complex could not be formed. This is in contrast to C5 binding by $EAC\overline{4,2,3}$, which proceeds in absence of C6 and C7 (26–28). But even in the classical immune hemolytic reaction, C6 and C7 greatly enhance C5 action by facilitating attachment of activated C5 molecules to the cell surface (18). Thus, it appears most probable from this and other studies (18, 26) that C5, C6, and C7 constitute a functional unit, although under specified conditions C5 can act independently of C6 and C7.

Comparing the effect of C5-C9 on E and on $EAC\overline{4,2,3}$ present in the same reaction mixture disclosed a large quantitative difference in lysis of the two cell types. Whereas under otherwise identical conditions lysis of $EAC\overline{4,2,3}$ proceeded with nanogram amounts of C5, lysis of E required microgram quan-

tities. This difference may indicate that successful binding of activated C5,6,7 is more likely to occur in the proximity of the activation site ($\overline{C4,2,3}$) than at some distance from it. In agreement with this assumption, the degree of lysis of E was found to be proportional to cell concentration, i.e., inversely proportional to the average distance between trigger and target cells. If nonimmune cytolysis should occur in vivo, the prevailing conditions would be more favorable than the optimal conditions employed in these experiments; in vivo the local cell concentration is generally high and so is the concentration of complement proteins in plasma.

Binding of C5,6,7 to target cells (E) after activation by trigger cells (EAC $\overline{4,2,3}$) requires transfer of the activated components. Transfer of C5,6,7 from the site of activation on one cell to the site of binding on another cell is distinct from any other known or postulated transfer phenomenon of complement components. It is reminiscent of the transfer of activated C3 (1) which was deduced from electron microscope evidence (29). C3 transfer, however, appears to occur only between an activation site ($\overline{C4,2}$) and a receptor site located on the same cell. C3 fails to achieve binding to a cell following activation in the fluid phase or at the surface of an adjacent cell. The C5,6,7 transfer is also distinct from transfer of C5 from $\overline{EAC4,2,3}$,5 to $\overline{EAC4,2,3}$, which Shin et al. (28) postulated for guinea pig complement. Here, a C5 molecule which was previously bound to a $\overline{C4,2,3}$ receptor site is assumed to transfer to other $\overline{C4,2,3}$ sites, including those located on other cells. A completely different type of reaction is the well documented transfer of C1 between antibody sites of the same or of different cells (30). This process is due to the relatively weak binding of C1 to immunoglobulins and to the stability of the C1 combining site. In contrast, the binding potential of other activated complement proteins, including C5,6,7, is labile; however, once binding has occurred the bond is usually much firmer than that of C1. Thus, the ability of C5,6,7 to transfer from the activation site (trigger cell) to a relatively distant binding site (target cell), suggests that the half-life of the activated combining site is not as short as in the case of C3. The experimental conditions which allowed effective transfer of C5,6,7 between trigger and target cells permit estimation of the maximal life span of the C5,6,7 combining potential. The inflection point of the ^{51}Cr release curve in Fig. 3 was determined by extrapolation of the two straight portions of the curve. It corresponds to a reaction volume of 0.3 ml. Since the total number of cells present was 10^8, the average distance (d) between the cells in suspension was calculated to be 2.6×10^{-4} cm, assuming a mean diameter for sheep erythrocytes of 4×10^{-4} cm. The approximate time (t) required for the activated trimolecular complex, (C5,6,7) to diffuse over the distance d is: $t = d^2/2D = 0.1$ sec. For D, the diffusion coefficient of (C5,6,7) at 37°C, a value of 3.3×10^{-7} cm^2/sec was assumed. It may be inferred from these considerations that the maximal life span of the activated binding site of C5,

6,7 is smaller than or equal to 0.1 sec, and that this permits the activated complex to react with receptors which are separated from the site of activation by maximally 2.5 μ.

Although most of the work has been done with sheep erythrocytes, similar results were obtained with normal human E and particularly with PNHE. The latter are known to be considerably more susceptible to lysis by complement than normal cells (31). This greater susceptibility can in part be explained in this study by the ability of PNH cells to bind a larger number of C5 molecules than normal human red cells. In addition, PNH cells exhibited a marked susceptibility to lysis triggered by cobra factor. This protein had previously been shown to combine with a β-globulin when added to serum and to form an enzymatically active complex which is able to cleave C3 in a manner analogous to $\overline{C4,2}$ (2). Recently, Pickering et al. (3) and Ballow and Cochrane (4) demonstrated that cobra factor could induce lysis of unsensitized sheep or guinea pig erythrocytes by autologous serum. It is probable that the cobra factor–β-globulin complex can substitute for the $\overline{C4,2}$ enzyme and thus activate in typical fashion the late-acting complement proteins. It is further probable that cobra factor analogs occur in the mammalian organism which are capable of activating the β-globulin in vivo. Similarly, formation of the $\overline{C4,2}$ enzyme might occur in vivo through antibody-independent activation of C1 which has been achieved in vitro by plasmin, trypsin (32), and kallikrein (33). Thus, both mechanisms might conceivably be operative in complement-dependent nonimmune cytolysis in vivo.

The concept emerging from this work may be formulated as follows. The C5,6,7 complex following enzymatic activation of its combining site can transfer to a receptor site on the surface of nonsensitized cells which contain no measurable amounts of either C2, C3, or C4. Bound C5,6,7 can then react with C8 and C9, following which the cell undergoes lysis. The $\overline{C4,2,3}$ enzyme can act either from the fluid phase or from the surface of another cell. Since C8 and C9 are unable to cause lysis of E in the presence of $EAC\overline{4,2,3},5,6,7$,[3] it appears that direct attack of complement on a target membrane cannot start later than at the C5,6,7 step. These findings allow an operational distinction between an activation mechanism of complement, consisting of C1,2,3 and C4, and an attack mechanism comprising C5,6,7,8 and C9.

SUMMARY

A new pathway of complement-mediated hemolysis has been described. It is independent of antibody and does not require binding of the first four complement components to the target-cell surface. The actual attack of the target cell begins with the attachment of C5, C6, and C7. The binding reaction is catalyzed by $\overline{C4,2,3}$, an enzyme which may be formed in cell-free solution.

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[3] Manni, J. A., and H. J. Müller-Eberhard. Manuscript in preparation.

$C\overline{4,2,3}$ may effect binding of C5,6,7 by acting from the fluid phase or from the surface of another cell to which it is specifically bound $(EAC\overline{4,2,3})$. In either case, the resulting product is EC5,6,7 which is susceptible to lysis by C8 and C9.

Erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH) were particularly susceptible to lysis by the above described mechanism. PNH cells, but not normal human erythrocytes, could also be lysed through activation of complement by cobra factor. These observations allow the operational distinction of an activation and an attack mechanism of complement.

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