Mechanisms of Immune Lysis of the Red Cells in Hereditary Erythroblastic Multinuclearity with a Positive Acidified Serum Test and Paroxysmal Nocturnal Hemoglobinuria

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A B S T R A C T The red cells of patients with hereditary erythroblastic multinuclearity with a positive acidified serum test (HEMPAS), a form of congenital dyserythropoietic anemia, and the cells of patients with paroxysmal nocturnal hemoglobinuria (PNH) are lysed more readily than normal cells by certain antibodies, notably cold agglutinins (anti-I) and complement. With some but not other examples of anti-I, HEMPAS and PNH cells adsorbed more antibody than normal cells. Equal quantities of adsorbed antibody bound equal quantities of the first component of complement (C1) to normal, PNH, and HEMPAS cells. However, for a given quantity of bound antibody and C1, much more of the fourth component of complement (C4) was bound to HEMPAS cells than to normal cells. This resulted in the binding of proportionately larger quantities of the third component of complement (C3) to these cells. The same amount of bound C3 was found on the membranes of normal and HEMPAS cells for a given degree of lysis. Hence, the marked increase in lysis of HEMPAS cells is due to the increased adsorption of antibody and/or increased binding of C4.

PNH cells bound the same amount of C4 per bound C1 as normal cells but bound more C3 than normal cells. However, the mean concentration of C3 on the membrane of PNH cells was one-third to one-fifth that on normal cells for a given degree of lysis. Hence, the increased lysis of PNH cells is due to the increased binding of C3 *and* increased hemolytic effectiveness of the bound C3.

INTRODUCTION

Normal red cells are not readily lysed by cold agglutinins and complement; lysis usually occurs only when relatively large amounts of antibody and/or complement are used. The red cells in at least two blood disorders appear to be more readily lysed than normal red cells by anti-I cold agglutinins and complement. The red cells of patients with paroxysmal nocturnal hemoglobinuria (PNH)[1] have been extensively studied and have been shown to consist of at least two populations, one of which is markedly sensitive to the lytic action of complement (1, 2). This sensitivity is apparent when complement is activated either by antibody or by the alternate (properdin) pathway (3–6). The latter mechanism of activation is responsible for the lysis of PNH cells in acidified normal serum (3, 4).

Crookston et al. reported that the red cells of patients with a form of congenital dyserythropoietic anemia characterized by multinucleated erythroblasts, are also lysed in acidified normal serum (7). They proposed the name hereditary erythroblastic multinuclearity with a positive acidified serum test, or HEMPAS; this

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[1] *Abbreviations used in this paper:* ACD, acid-citrate-dextrose solution; C1, C2, C3..., the first, second, third, etc. components of serum complement (a bar over the number indicates that the component is in its active form); CLS H_{50}, a unit of sensitivity to complement lysis, viz. the reciprocal of the dilution of complement 0.2 ml of which will lyse 50% of 2.2×10^7 sensitized red cells in a total volume of 1.5 ml; CoF, a factor derived from cobra venom which initiates the alternate pathway of C3 activation; EAC, red cells sensitized with antibody and coated with components of complement; HEMPAS, hereditary erythroblastic multinuclearity with a positive acidified serum test; PNH, paroxysmal nocturnal hemoglobinuria; VBS, veranol-buffered saline.

syndrome also has been classified as congenital dyserythropoietic anemia, type II, by Heimpel and Wendt (8). The lysis in normal serum appears to be initiated by an alloantibody (anti-HEMPAS) against an antigen detectable only on the red cells of HEMPAS patients (7, 9). HEMPAS cells are somewhat more sensitive than normal cells to lysis by anti-I and complement when antibody is in relative excess (10, 11).

The present studies were undertaken to define the mechanisms of increased lysis of HEMPAS and PNH cells by anti-I by analysis of their reactions with antibody and complement. They show that HEMPAS cells are more readily lysed than normal cells because of increased efficiency at two steps: (a some, but not all, examples of anti-I are bound to a greater extent by these cells, and (b more C4 is bound per fixed C1 molecule. The markedly abnormal complement-sensitive PNH cells (population III [12]) are more readily lysed than normal cells for two reasons: (a more C3 is fixed for a given amount of fixed-antibody, C1 or C4 and (b a greater degree of lysis occurs as a result of the fixation of a given amount of C3 to these cells.

METHODS

Red cells. Blood was obtained from 10 HEMPAS patients, from 6 obligate heterozygotes (parents of HEMPAS patients), from 10 patients with PNH, and from eight normal subjects (laboratory personnel). Blood was drawn aseptically into acid-citrate-dextrose solution (ACD) (6 vol blood to 1 vol ACD, NIH formula B) or into equal volumes of Alsever's solution, and stored at 4°C for up to 3 wk. At the time of testing, the cells were washed twice in veronal-buffered saline (VBS) and resuspended in VBS in a standard concentration of 2.2×10^8 cells/ml (1).

Antibodies. Blood containing cold-reactive antibodies was clotted and the serum removed at 37°C. Sera containing anti-I were obtained from eight patients with chronic cold agglutinin disease. Donath-Landsteiner antibody was obtained from two patients with paroxysmal cold hemoglobinuria (PCH): one with PCH after an acute viral infection (Hal.), and the other with chronic idiopathic immune hemolytic anemia (Jack.). Heterologous antibodies to red cell antigens were raised in rabbits after the intravenous injection of either whole red cells or boiled red cell stromata at daily intervals for 15 days. The antiserum was harvested 5 days after the last injection.

Purified cold-reactive antibody was prepared as follows: 1 vol of serum containing cold agglutinins was mixed with 5 vol of washed, packed, red cells from a group O, adult donor. The mixture was cooled at 0°C for 2 h with gentle agitation. The mixture was centrifuged at 0°C and the supernatant fluid was removed and replaced with an equal volume of VBS. The cells were resuspended at 37°C for 1 h, cooled at 0°C as before, and the supernatant fluid was removed and discarded. After the supernatant fluid was removed and replaced for the third time, the mixture was warmed and centrifuged at 37°C, and the cells were discarded. The supernatant fluid was passed through a Sephadex G-200 column, and the exclusion peak (containing the cold agglutinin) was concentrated by pressure dialysis. To label the antibody with ^{125}I, the iodine monochloride

technique of Hemlkamp, Goodland, Bale, Spar, and Mutschler was used (13). After this procedure, more than 98% of the radioactivity was precipitable by trichloroacetic acid, and over 95% of the radioactivity could be removed by repeated absorptions with normal adult red cells at 0°C. The purified IgM antibody showed a single precipitin line when tested by immunoelectrophoresis against goat anti-whole serum, rabbit anti-IgM, and rabbit anti-κ antisera.

Buffers. VBS containing Ca^{++}, Mg^{++}, and 1% gelatin was made by the method of Mayer (14).

EDTA. 0.1 M EDTA was made by the method of Frank, Rapp, and Borsos (15). For use, it was diluted to 0.015 M in VBS containing no Ca^{++} or Mg^{++}.

Complement components and intermediates. Partially purified first (C1) and second (C2) components of guinea pig complement, sheep cells sensitized with rabbit antibody to sheep red cell stromata, and components of guinea pig complement (EAC4) were made according to the methods given in Rapp and Borsos (16). A standard solution of the fourth component of complement and the antibody to it were obtained from Meloy Laboratories, Inc., Springfield, Va. The anti-C4 gave single lines on immunoelectrophoresis against whole human serum and against the C4 preparation.

Third component of complement (C3). Pure C3 was made from human serum by the method of Nilsson, and Müller-Eberhard (17) as modified by Logue, Rosse, and Gockerman (18). Antibody to C3 was made by injecting pure human C3 in Freund's complete adjuvant into the foot pads and subcutaneous sites of rabbits; antiserum was collected 3 and 6 wk later.

EAC43. Human red cells coated with complement components (at least C4 and C3) but lacking antibody were prepared by mixing equal volumes of a standard suspension of cells, anti-I in appropriate dilution, and fresh human serum as a source of complement. The mixtures were incubated at 0°C for 15 min, then at 37°C for 60 min. The mixtures were centrifuged and the proportion of cells lysed was determined from the optical density of the supernatant fluid at 412 nm. The surviving cells were washed in warm (37°C) 0.015 M EDTA and VBS, and were then resuspended at a concentration of 2.2×10^8/ml.

Tests for PNH and HEMPAS. The sucrose lysis test was performed by the method of Hartmann, Jenkins, and Arnold (19).

The cobra venom lysis test was performed by the method of Kabakçi, Rosse, and Logue (6).

The acidified serum lysis test was performed by the method of Dacie and Lewis (20).

The complement lysis sensitivity test was performed by the method of Rosse and Dacie (1) with the modification that complement, anti-I, and cells were mixed at 37°C, incubated at 0°C for 15 min, then rewarmed at 37°C. In this test, antibody (usually anti-I [Step.]) was present in excess and the amount of complement was varied. The relative sensitivity of the cells to complement lysis was expressed as the "complement lysis sensitivity titer" (CLS H$_{50}$) i.e., the reciprocal of the dilution of normal serum, 0.2 ml of which will lyse 50% of the cells in a total volume of 1.5 ml.

Absorption of antibody. Purified cold agglutinins (anti-I) labeled with ^{125}I were diluted in VBS; 0.1 ml of antibody was mixed with 0.1 ml of standard red cell suspension. After 2 h at 0°C, the mixture was centrifuged at 0°C and the supernatant fluid was carefully removed with a micropipette. The radioactivity adhering to the cells was determined in a well-type gamma-ray spectrometer. The

amount of antibody protein absorbed to the cells was calculated by comparing the radioactivity present on the cells with that of a known quantity of the same labeled, purified antibody.

Lysis by antibody in the presence of excess complement. Purified anti-I was prepared in twofold falling dilutions from 0.1 mg/ml; 0.4 ml of each dilution was mixed with 0.2 ml of fresh, normal, compatible serum as a source of complement, and 0.1 ml of standard red cell suspension. The mixtures were placed at 0°C for 15 min and at 37°C for 60 min. 4 ml of VBS was added, and the cells were removed by centrifugation. The optical density of the supernatant fluid at 412 nm was determined and the percent lysis was calculated.

To relate the amount of lysis to the amount of antibody adsorbed during the cold phase of the test, duplicate samples were prepared containing each dilution of ^{125}I-labeled antibody with red cells and fresh normal serum. After incubation for 30 min at 0°C, one set of samples was measured for radioactivity. The other set of samples was incubated at 37°C for 30 min and the amount of lysis was measured.

Fixation of the first component of complement ($C\bar{1}$) by cold agglutinins. The amount of $C\bar{1}$ bound to red cells by antibody was determined by the $C\bar{1}$ fixation and transfer test of Borsos and Rapp (21) as modified by Rosse and Sherwood (22). Equal volumes (0.1 ml) of a standard red cell suspension and of anti-I (Step. and Tur.), appropriately diluted, were mixed with 0.2 ml of partially purified $C\bar{1}$ and incubated for 60 min at 0°C, and the excess $C\bar{1}$ was then removed by washing. The bound $C\bar{1}$ was released and measured by the hemolytic assay of Borsos and Rapp (23). To determine the amount of $C\bar{1}$ bound per unit of adsorbed antibody, red cells were mixed with ^{125}I-labeled anti-I and partially purified $C\bar{1}$, and left at 0°C for 1 h. Duplicate series were prepared; the cells in both series were washed as for the $C\bar{1}$ fixation and transfer test. The amount of radioactive antibody adsorbed on the cells of one series was determined. The amount of $C\bar{1}$ bound to the cells of the other series was determined by the $C\bar{1}$ fixation and transfer test.

Measurement of the fourth component of complement (C4) bound to human red cells

Membrane-bound C4 was determined by a modification of the method of Borsos and Leonard for the quantitation of C3 by absorption of anti-C3 (24). The fourth component of complement (C4) was bound to the membranes of sheep cells by the method used by Borsos and Leonard (24) to bind C3, and the amount of anti-C4 required to lyse cells in the presence of guinea pig complement was determined. This quantity of anti-C4 was incubated with membranes or cells to which C4 was bound (EC43) and the relative amount of anti-C4 remaining after incubation was determined by lysis of the $E^{sh}C43$. The degree of antibody absorption was calibrated using known quantities of C4. Assuming a molecular weight of 250,000 daltons for C4, the number of molecules of C4 bound per cell was calculated.

C2 adsorption by EC43. The relative concentration of C4 bound to normal, HEMPAS, and PNH red cells was determined by a modification of the technique of Sitomer, Stroud, and Mayer. (25) which is based upon the adsorption of C2 from solution by cells coated with C4 but lacking C1 (EC43). EC43 cells (4.4×10^8/ml) were incubated

at room temperature for 20 min with equal volumes of three dilutions of a known concentration of functionally purified C2. The amount of C2 in the solution before and after adsorption was determined by the method of Borsos, Rapp, and Mayer (26) and the results were calculated by the method of Hoffman and Meier (27). The relative number of molecules of C2 bound to the membrane was determined by the method of Sitomer et al. (25) based upon the Langmuir absorption isotherm (28). The estimate of C2 by these techniques is a minimum estimate since not all C2 in solution is detected by guinea pig C-EDTA.

Measurement of the third component of complement (C3) bound to human red cells

Fixation of C1 by anti-C3. The amount of C3 bound to red cells after reaction with cold agglutinins ($E^{Hu}C43$) was determined by a modification of the $C\bar{1}$ fixation and transfer test, using anti-C3 to fix $C\bar{1}$ (18).

Consumption of anti-C3. The amount of membrane-bound C3 was also determined by a modification of the method of Borsos and Leonard (24). Human red cells coated with C3 were incubated with an amount of anti-C3 capable of lysing 60–80% of sheep cells coated with human complement components ($E^{sh}C43$) in the presence of guinea pig complement. The absorption of anti-C3 from solution was detected by reduction in the lysis of the coated sheep cells. The amount of anti-C3 remaining after absorption with C3-coated human red cells was compared with the amount of anti-C3 remaining after incubation with known concentrations of purified C3.

To determine the amount of C3 and/or C4 bound per unit of adsorbed antibody, duplicate series of red cells, serial dilutions of ^{125}I-labeled cold agglutinins, and fresh normal serum were incubated at 0°C for 30 min. One series was then centrifuged and the amount of radioactivity adsorbed to the cells was determined. The other series was incubated at 37°C for 1 h and the amount of C3 and/or C4 bound to the cells was determined.

The kinetics of C3 and C4 binding to normal and HEMPAS cells. To investigate the kinetics of antibody dissociation and C4 and C3 binding during warming, equal volumes of normal or HEMPAS cells in standard suspension were mixed with equal volumes of radiolabeled antibody and fresh human serum in appropriate dilution. For these studies, dilutions of antibody were selected so that, at equilibrium, about the same amount of antibody was present on normal and HEMPAS cells. The flask was then gradually warmed at the rate of 1°C/min. At intervals of time, 0.2-ml aliquots were removed, rapidly filtered with suction through Whatman glass fiber paper (gf/c)[a] and the cells adherent to the filter were washed with 1 ml ice-cold VBS. The radioactivity due to antibody adsorbed to the cells adherent to the filter was later determined in a well-type gamma-ray spectrometer. At the time that the sample was removed for antibody analysis, a second sample was removed and the cells were lysed in cold 0.015 M EDTA in water. The resultant stromata were washed twice in isotonic EDTA-VBS and twice in VBS, and the amount of C3 and C4 present on the membranes was determined by the method of Borsos and Leonard (24).

Inhibition of antibody adsorption by membrane-bound C3. To determine the degree to which membrane-bound C3 inhibited adsorption of anti-I, C3 was bound to normal

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[a] Obtained from H. Reeve Angel Co., Clifton, N. J.

and heterozygous and homozygous HEMPAS red cells by exposing the cells to anti-I (Step., Viv., and Tur.) and fresh normal serum (as a source of complement) at 0°C for 15 min, and then at 37° for 1 h. Those cells which remained unlysed were washed and the relative amount of C3 bound was determined by the $\overline{CI}$ fixation and transfer technique using anti-C3.

The C3-coated cells were then exposed to ^{125}I-labeled anti-I, and the amount of antibody adsorbed was determined. In the same way, cells not coated with C3 were exposed to radiolabeled anti-I to determine the maximum uptake of antibody. The percent inhibition was calculated from the formula: $[(cpm_o - cpm_T)/cpm_c] \times 100$, where cpm_T = counts per minute on the test cells and cpm_c = counts per minute on cells not coated with C3. The "inhibition ratio" was calculated by dividing the percent inhibition by the number of molecules of $\overline{CI}$ bound to the cells by anti-C3.

Similar experiments were performed using cells coated with C3 in the absence of antibody. These cells were prepared by incubating a 5% suspension of normal, or heterozygous, or homozygous HEMPAS cells in 9 vol of compatible normal serum diluted 1 in 20 in isotonic sucrose, and incubating at room temperature for 60 min.

RESULTS

Sensitivity to lysis by complement. The cells of eight patients with HEMPAS, four obligate heterozygotes, two other family members, eight normals, and 10 patients with PNH were tested for relative susceptibility to lysis by complement in the complement lysis sensitivity test (1). In this test, antibody was present in excess and the amount of complement present was varied. Anti-I (Step.) was used as the sensitizing antibody in most instances.
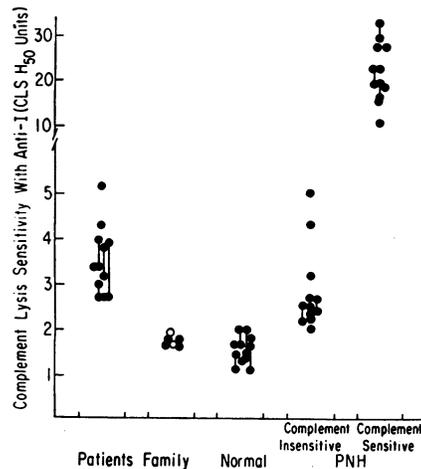

Figure 1 Results of complement lysis sensitivity tests on HEMPAS patients, family members, PNH patients, and normal controls. Duplicate tests on different samples from the same patient are connected by a line. Obligate heterozygotes among family members are shown in open circles.

Table I

The Relationship Between Adsorbed Anti-I and Lysis of Normal, HEMPAS, and PNH Cells

Anti-I antibody	Cells		Fraction anti-I adsorbed	Molecules anti-I/cell × 10⁻³	
				10% lysis	50% lysis
Tur.	Normal	M. C.	0.15	31.0	—
		G. L.	0.085	25.3	—
	HEMPAS Heterozygous	H. F.	0.17	10.33	63.84
	Homozygous	M. F.	0.30	2.74	10.9
		L. F.	0.33	2.13	7.9
		C. L.	0.38	1.83	5.5
	PNH	A. G.	0.21	0.45	2.43
Step.	Normal	M. C.	0.24	2.25	6.20
		D. S.	0.26	1.16	4.86
	HEMPAS Heterozygous	H. F.	0.27	0.94	3.40
	Homozygous	M. F.	0.29	0.33	1.09
		L. F.	0.26	0.18	0.55
		C. L.	0.26	0.12	0.39
	PNH	A. G.	0.26	—	0.091
Viv.	Normal	M. C.	0.12	37.7	—
		G. L.	0.13	36.5	—
	HEMPAS Homozygous	M. F.	0.26	6.69	18.24
		L. F.	0.26	1.82	6.38
		C. L.	0.33	1.82	6.38
	PNH	A. G.	0.21	0.36	1.82

The cells of all patients with HEMPAS were more susceptible than normal cells to lysis by complement; one-half to one-third the amount of complement was required to lyse 50% of the HEMPAS cells as compared with normal cells (Fig. 1). In normal subjects and HEMPAS patients, a single population of cells with respect to sensitivity to lysis by complement was found (10).

The PNH blood samples contain two red cell populations, one similar to normal cells (population I) and another highly sensitive to the effect of complement (population III) (1, 12). Blood from patients with very large proportions of population III (75% or more) was used in the studies.

The degree of sensitivity of HEMPAS red cells to immune lysis was more striking when complement was present in constant excess and the amount of antibody was limiting. HEMPAS cells required 1/10 to 1/15 the amount of antibody for the same degree of lysis as normal cells, regardless of the anti-I used (Table I and Fig. 2a, b). Three obligate HEMPAS heterozygotes could be distinguished from normal subjects by this technique, but the cells of one parent of a HEMPAS patient appeared normal.
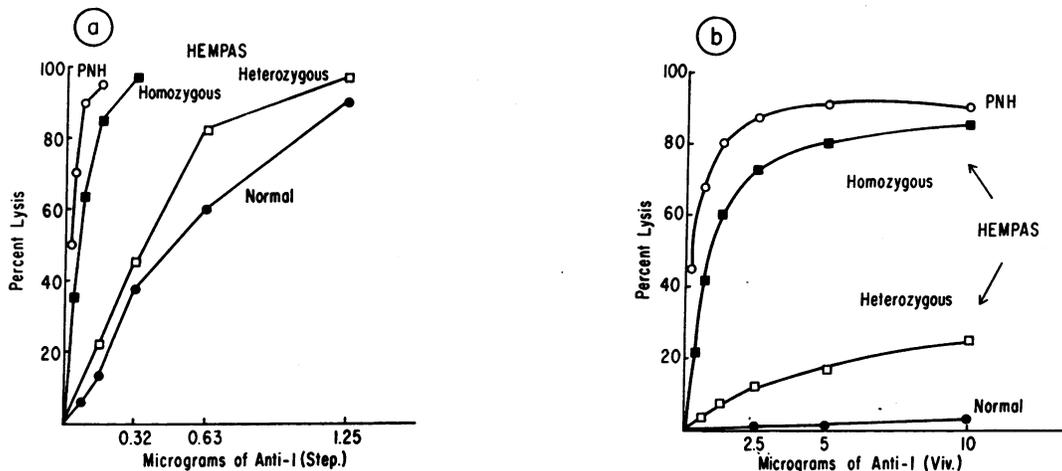
FIGURE 2 Lysis of normal, PNH, and homozygous and heterozygous HEMPAS cells by anti-I (Step.) (2a) and anti-I (Viv.) (2b), when complement was present in relative excess and the concentration of antibody was limiting.

HEMPAS cells were also lysed to a greater extent than normal cells by two cold-reactive antibodies, anti-i and the Donath-Landsteiner antibody, and two warm-reacting antibodies made in rabbits by injection of whole cells or boiled stromata of normal red cells (Table II). Tests with the heterologous antibodies were performed at 37°C throughout.

Adsorption of anti-I by normal, HEMPAS, and PNH cells. The adsorption of eight examples of anti-I on normal, HEMPAS, and PNH red cells is shown in Table III. Two examples of anti-I (Cass., Step.) were adsorbed nearly to the same degree on all three types of cells. The other examples were adsorbed from 1.5 to 3.5 times as much on HEMPAS cells as on normal cells.

When one example from each category (Step. and Tur.) was used for further tests, it was found that much less adsorbed antibody was required to lyse HEMPAS

and PNH cells than to lyse normal cells (Fig. 3a, b). Using one example of anti-I (Tur.), all three types of cells bound larger amounts of antibody for a given degree of lysis than when another anti-I (Step.) was used.

Fixation of complement by antibody

Fixation of the first component of complement ($C\bar{1}$). When red cells were exposed to radiolabeled anti-I and partially-purified $C\bar{1}$, equal amounts of $C\bar{1}$ were fixed per molecule of adsorbed antibody on normal, HEMPAS, and PNH cells (Table IV).

The fixation of C4. The results of the determination of membrane-bound C4 related to the amount of adsorbed anti-I on normal, PNH, and HEMPAS cells are shown in Fig. 4a and b. For a given amount of adsorbed antibody, more C4 was bound to HEMPAS than to normal cells. The amount of C4 bound to PNH cells per molecule of adsorbed antibody was the same as that bound to

TABLE II
Lysis of Normal, HEMPAS, and PNH Red Cells by Antibodies Other than Anti-I

| Antibody | Dilution | Percent lysis | | |
		Normal	HEMPAS	PNH
Anti-i				
Ho.	1/40	0	35	0
Den.*	1/100	0	62	5
Donath-Landsteiner				
Jack	1/5	10	72	85
Hal.	1/5	15	58	76
Rabbit anti-red				
cell stromata‡	1/27	17	93	79
Rabbit anti-red cell‡	1/243	0.4	62	64

* Kindly supplied by Dr. D. H. Cowan.
‡ Tests performed at 37°C throughout.

TABLE III
Adsorption of Several Examples of Radiolabeled Anti-I to Normal, HEMPAS, and PNH Red Cells

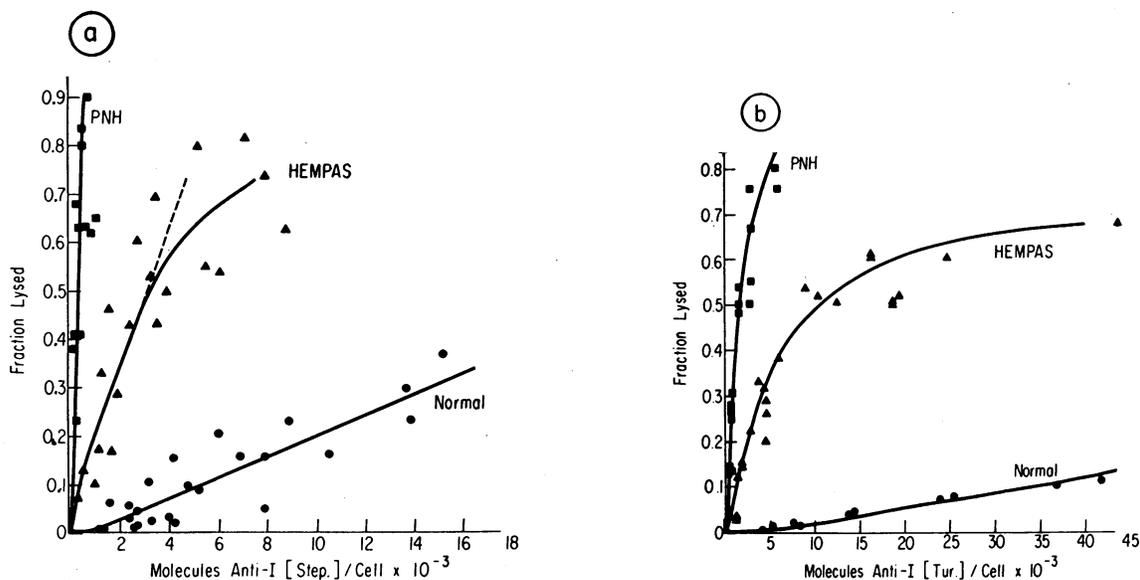| Antibody | Fraction adsorbed on cells | | |
	Normal	HEMPAS	PNH
Bos.	0.16	0.25	0.18
Cass.	0.13	0.18	0.16
Gil.	0.12	0.40	0.20
Gre.	0.13	0.33	0.17
Per.	0.24	0.68	0.41
Step.	0.68	0.73	0.74
Tur.	0.12	0.33	0.21
Viv.	0.23	0.50	0.37

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FIGURE 3 The lysis of normal (●), PNH (■), and homozygous HEMPAS cells (▲) related to the amount of adsorbed anti-I (Step.) (3a) and anti-I (Tur.) (3b). Complement is present in relative excess.

normal cells. Less C4 was adsorbed to all cells per molecule of anti-I (Tur.) than of anti-I (Step.).

The adsorption of C2. EC43 was made with normal, PNH, and HEMPAS cells, and the amount of membrane-bound C4 was estimated by C2 adsorption. The amount of C2 adsorbed to all these cells was found to be proportional to the amount of C4 present on the cells (Table V).

Fixation of the third component of complement (C3) and its relationship to the amount of lysis obtained. When red cells were exposed to radiolabeled anti-I (Tur., Step.), and fresh normal serum (as a source of complement) and both adsorbed anti-I and bound C3 were measured, the results shown in Fig. 5a and b were obtained. For a given amount of antibody, both HEMPAS and PNH cells bound considerably more C3

than normal cells. However, the amount of C3 bound per molecule of C4 was the same for normal and HEMPAS cells, whereas five to seven times as much C3 was bound per molecule of C4 by PNH cells (Table VI).

The relationship between the amount of membrane-bound C3 and the degree of lysis obtained is shown in Figs. 6a and b. When anti-I (Step.) was used, the amount of C3 bound at a given degree of lysis was about the same for normal and HEMPAS cells, suggesting that the later steps of the complement sequence were equally inefficient in effecting the lysis of these two kinds of cells. When C3 was fixed to the membrane by anti-I (Tur.), less C3 was fixed for a given degree of lysis to HEMPAS cells than to normal cells. With either anti-I, PNH cells had one-third to one-fifth as much fixed C3 for a given degree of lysis as either normal or HEMPAS cells. This finding indicates that C3

TABLE IV

Fixation of Antibody and $C\overline{1}$ to Normal, HEMPAS, and PNH Cells

Anti-I	Cells	Molecules anti-I adsorbed	Molecules $C\overline{1}$ fixed	Ratio $C\overline{1}$/anti-I
Step.	Normal	18,850	1,840	0.097
	HEMPAS	19,230	2,035	0.10
	PNH	20,970	1,930	0.092
Tur.	Normal	20,055	2,100	0.105
	HEMPAS	62,300	5,880	0.094
	PNH	27,623	2,550	0.092

TABLE V

Adsorption of C2 by C4 on Normal, HEMPAS, and PNH Cells

Cell	Adsorbed C4	Adsorbed C2	Ratio C2/C4
	molecules/cell	*SFU/cell**	
Normal	2,400	465	0.194
HEMPAS	9,400	1,333	0.1420
PNH	5,000	606	0.121

* SFU, site-forming unit (27), estimated from the Langmuir isotherm (see text).

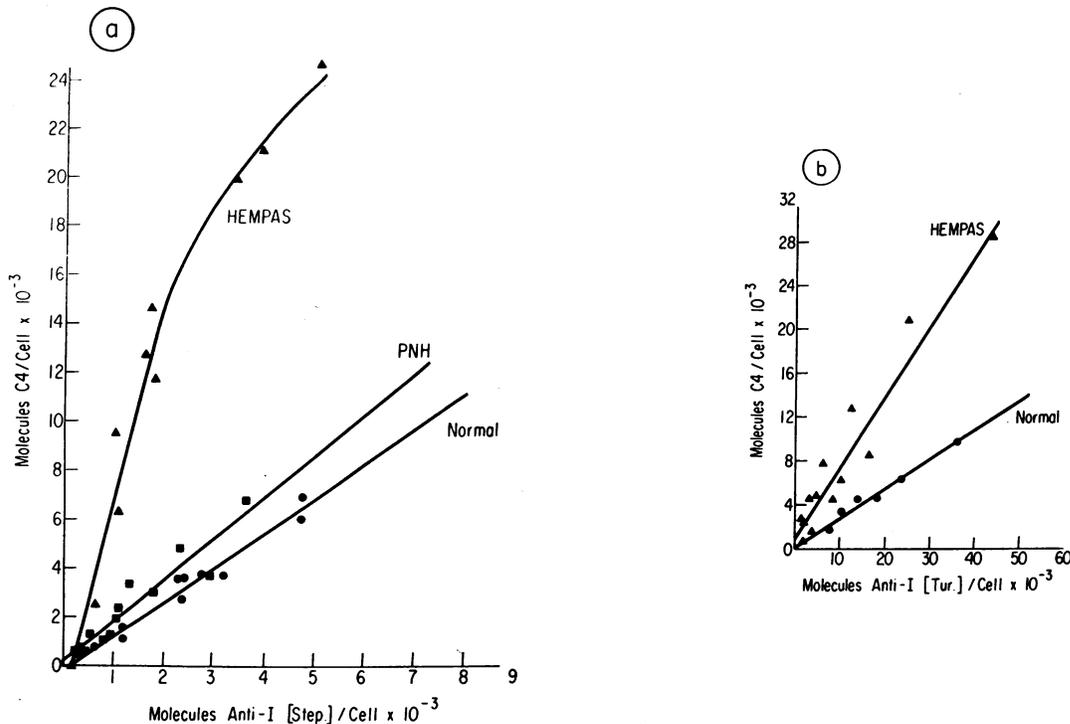36   *W. F. Rosse, G. L. Logue, J. Adams, and J. H. Crookston*

FIGURE 4   The amount of fixed C4 related to the amount of adsorbed anti-I on normal (●), HEMPAS (■), and PNH (▲) cells. (a) Anti-I (Step.), (b) Anti-I (Tur.).

and/or the steps of complement activation following C3 are more efficient on PNH cells (Table VI).

The kinetics of C3 fixation during warming. As shown in Fig. 7a and b, the rate of elution of anti-I from normal and HEMPAS cells during warming was not significantly different. Further, the temperature at which C4 and C3 were bound was not different. However, the rate at which C4 and C3 were bound, and the total amount of these components which was bound, was markedly greater for HEMPAS cells than normal cells.

Inhibition of antibody adsorption by membrane-bound C3. When C3 was bound to the membrane of HEMPAS and normal cells by anti-I, the degree to which it inhibited antibody adsorption differed for each example of anti-I (Viv., Step., Tur.) but with each antibody, inhibition was less for HEMPAS cells than for normal cells. HEMPAS heterozygotes showed intermediate values (Fig. 8). When a medium of low ionic strength was used to bind C3 to membrane in quantities equal to that bound by antibody, the C3 did not inhibit the uptake of anti-I.

The lysis of HEMPAS and PNH cells in normal acidified serum. Acidified serum known to contain the alloantibody, anti-HEMPAS, was tested before and after absorption with normal, HEMPAS, or PNH red cells. The amount of membrane-bound C3 and the de-

gree of lysis were determined (Table VII). In the unabsorbed serum, both PNH and HEMPAS cells fixed considerable amounts of C3 and were lysed. However, serum which had been absorbed with HEMPAS cells before the incubation, no longer fixed C3 to HEMPAS cells and did not cause lysis. Absorption of serum with normal, PNH, and HEMPAS cells did not affect C3 fixation to, or lysis of, PNH cells.

TABLE VI
Efficiency of Complement Steps in Lysis of Abnormal Cells by Anti-I (Step.)

	Normal	HEMPAS	PNH
Fraction of antibody adsorbed	0.75	0.71	0.82
Molecules C1 per molecule anti-I	0.10	0.11	0.12
Molecules C4 per molecule C1	12	59	12.5
Molecules C3 per molecule C4	5.32	3.6	31.2
Lytic events per C3 molecule/($\times 10^{-6}$)*	3.3–7.5	5.0–10.0	22.7–25.5

* Assuming a single lytic event is sufficient for lysis.
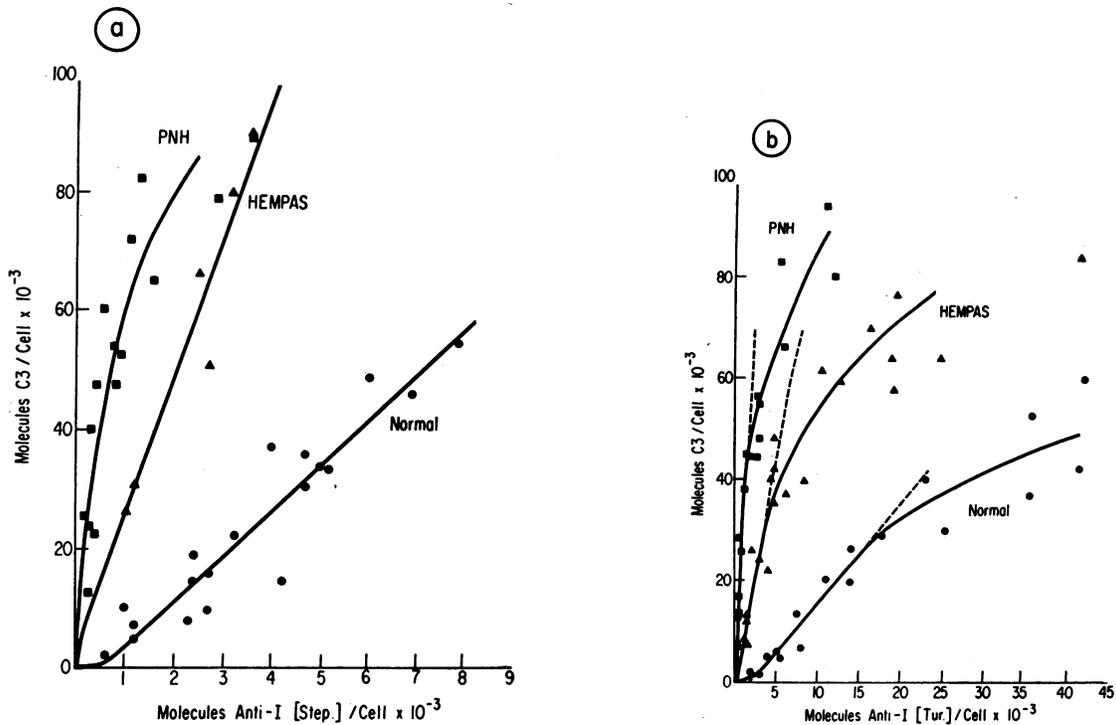
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FIGURE 5 The amount of fixed C3 related to the amount of adsorbed anti-I on normal (●), PNH (■), and HEMPAS (▲) cells. (a) Anti-I (Step.), (b) Anti-I (Tur.).

The acidified normal serum containing anti-HEMPAS was used to measure the fixation of C4 and C3 to normal, PNH, and HEMPAS cells. C4 was fixed to HEMPAS cells but not to PNH or normal cells (Table VIII).

The fixation of C3 to normal, HEMPAS, and PNH cells following activation of the alternate pathway of complement activation by cobra venom factor. Normal, PNH, and HEMPAS red cells were incubated with
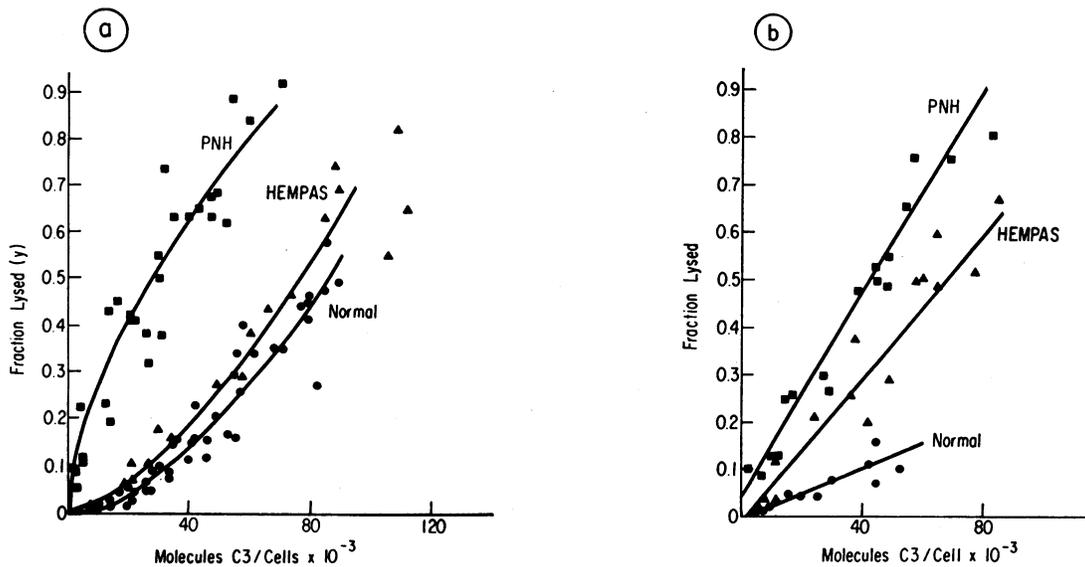

FIGURE 6 The amount of C3 bound to normal (●), PNH (■), and HEMPAS (▲) cells related to the degree of lysis obtained. (a) Anti-I (Step.), (b) Anti-I (Tur.).

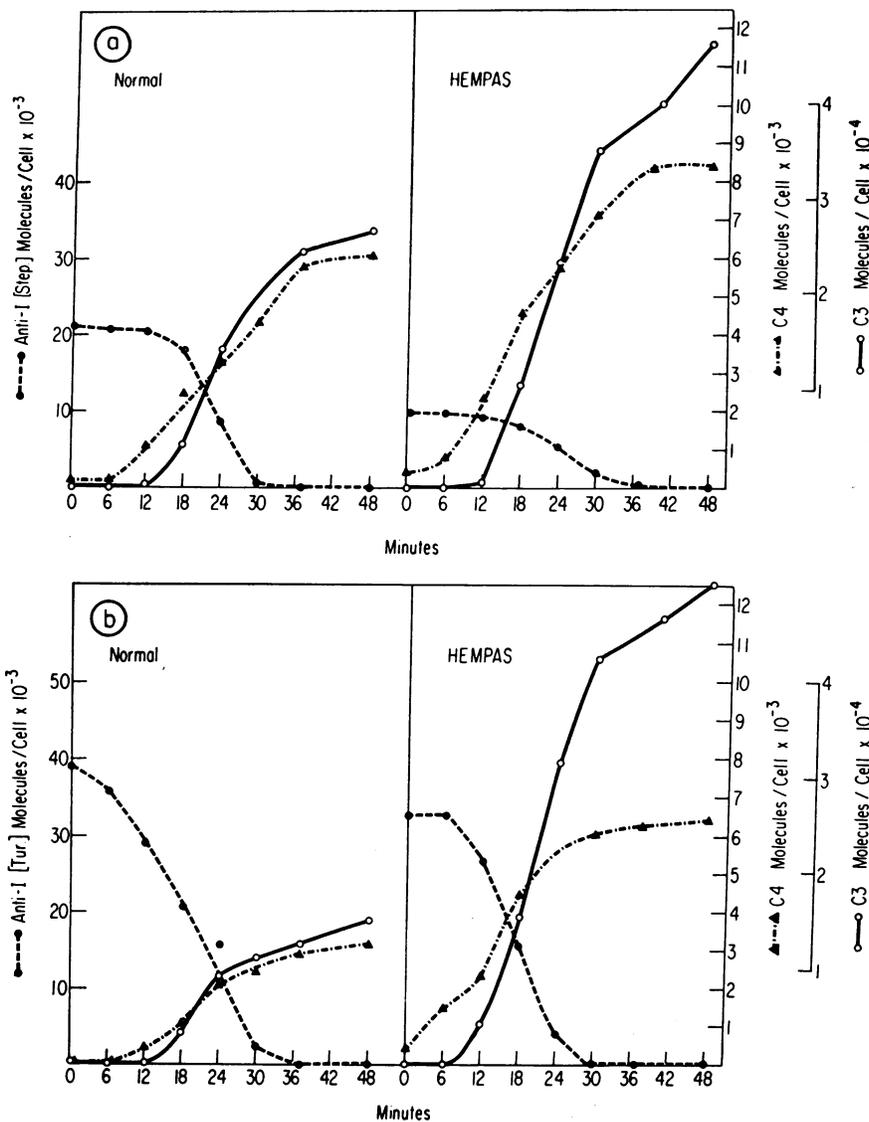38   W. F. Rosse, G. L. Logue, J. Adams, and J. H. Crookston

FIGURE 7 The rate of antibody elution and C4 and C3 fixation on normal and HEMPAS cells during warming. The mixture was warmed at the rate of 1°C per minute to 37°C and held at that temperature to the end of the incubation. (a) Anti-I (Step.), (b) Anti-I (Tur.).

fresh serum, serum which had been activated by cobra venom factor, and 0.015 M EDTA. The fixation of C3 to the membranes and the degree of lysis was determined. C3 was fixed only to the PNH cells and only these cells were lysed (Table VIII).

DISCUSSION

The red cells of patients with HEMPAS are unusually susceptible to immune lysis when complement is activated by cold agglutinins. Lewis, Grammaticos, and Dacie suggested that the difference between normal cells and HEMPAS cells in the complement lysis sensitivity

test of Rosse and Dacie was, "mainly or entirely" due to increased antibody adsorption by the abnormal cells (11). In the present investigation, HEMPAS cells did adsorb more antibody than normal cells from some, but not all examples of anti-I. However, increased uptake of antibody was not the only cause of increased lysis and, in fact, appeared to play no role in the increased lysis of HEMPAS cells with some examples of anti-I which were adsorbed in normal amounts (Cass. and Step. in Table III). Relatively much more C4 and, as a consequence, more C3 were bound to HEMPAS cells than to normal cells by the same amount of ad-

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Lysis of and C3 Fixation to Normal, HEMPAS, and PNH Red Cells by Acidified Normal Serum
Before and After Absorption with these Cells

| | Unabsorbed serum* | | Serum absorbed with cells | | | | | |
| | | | Normal | | HEMPAS | | PNH | |
Cells	% lysis	C3‡	% lysis	C3‡	% lysis	C3‡	% lysis	C3‡
Normal	0	1.0	0	0.36	0	0.36	0	0.36
HEMPAS	18.8	39.2	5.3	10.14	1.1	0.70	7.1	14.84
PNH	52	47.1	45.8	39.03	45.0	38.70	44.2	33.52

* Known to contain the alloantibody, anti-HEMPAS (7, 9).
‡ Molecules of C3/cell × 10⁻³.

sorbed antibody (Figs. 4 and 5). This difference appears to explain in large part the increased lysis of these abnormal cells.

The mechanism by which a given amount of antibody is able to initiate the binding of more complement sequences on HEMPAS than on normal cells is not clear. In the complement sequence, there are several steps which amplify the reactions; that is, a small amount of one component effects the binding of a large amount of the succeeding component. Thus, small amounts of C1 activate large amounts of C4 (29), and it appears to be this step which is exaggerated on HEMPAS cells since the same amount of C1, but a larger amount of C4 was fixed by a given amount of adsorbed antibody. The fixation of C3 by $\overline{C42}$ complexes is also thought to be an amplifying step; this step did not appear to be exaggerated on HEMPAS cells (Table VI).

Since anti-I, and hence C1, are dissociated from the cells when they are warmed to 37°C (30), most of the

fixation of C4 and C3 occurs during the warming phase. We therefore investigated whether more antibody might be present on HEMPAS cells at any point during the warming phase to account for the marked increase in fixation of complement components. However, the rate of dissociation of anti-I from HEMPAS cells was not different from normal (Fig. 7a, b). The resultant marked increase in membrane-bound C4 and C3 could not be attributed to an increased amount of antibody on HEMPAS cells at any time during warming. Further, increased lysis was seen with rabbit anti-red blood cell antibody when the reaction was performed at 37°C (Table II), thus ruling out any effect of differential dissociation of antibody during warming.

Evans, Turner, and Bingham (31) have shown that C3 bound to the red cell membrane by anti-I inhibits the adsorption of anti-I. The present studies show that C3 bound in large amounts to HEMPAS cells was less inhibitory to antibody adsorption that C3 bound to normal cells (Fig. 3). This difference could explain the increased binding of C4 by HEMPAS cells. During the interaction of antibody and complement on the membrane of normal cells, as more C3 accumulates, adsorption of antibody may be inhibited, thus limiting the number of active $\overline{C42}$ complexes which can be generated.
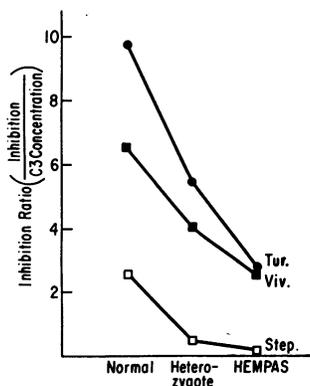

FIGURE 8 The inhibition of anti-I adsorption by membrane-bound C3 on normal and homozygous and heterozygous HEMPAS cells. The inhibition ratio was calculated by dividing the percentage of the inhibition of antibody adsorption by the concentration of C3 on the cell and multiplying the result by 100.

TABLE VIII
Fixation of C4 and C3 to Normal, HEMPAS, and PNH
Red Cells by Acidified Serum and Cobra
Venom Factor-Activated Serum

Test	Cells	C4	C3	Lysis
		molecules/cell	molecules/cell	%
Acidified	Normal	0	0	0
normal sera	HEMPAS	4,450	20,000	17.7
	PNH	0	25,720	42.3
CoF-activated	Normal	—	0	0
	HEMPAS	—	0	0
	PNH	—	2,000	47.0

This in turn may limit the total number of C3 molecules which can be bound to cells. In contrast, C3 bound to HEMPAS cells is less inhibitory to antibody adsorption and may permit the binding of more antibody and the generation of more $\overline{C42}$ complexes on HEMPAS than on normal cells. This mechanism does not appear to play a major role when the amount of C3 on the membrane is small (Fig. 7).

The fact that many molecules of C3 are bound to the membrane of red cells surviving lysis by antibody and complement (18, 32) suggests that some inefficient step(s) interfere with the ability of initiated complement sequences to go to completion. Some of this apparent inefficiency may be due to the placement of the bound C3. At the present time, little is known about the spatial or chemical characteristics which permit some, but not other, bound C3 molecules to produce "hemolytically active" sites. Evidence has been adduced to indicate that only those C3 molecules in close approximation to C42 sites may partake in the cleavage of C5 (33). Further, cell-bound C3 molecules are rapidly inactivated by enzymatic cleavage by C3b inactivator (KAF) (34, 35).

Whatever the reasons, the same proportion of bound C3 molecules bound to HEMPAS red cells are "hemolytically effective" as that bound to normal red cells, since, for a given degree of lysis, the same amount of C3 is bound to both types of cells. Hence, the increased lysis of HEMPAS cells by a given concentration of antibody and serum as a source of complement is largely due to the increased number of initiated complement sequences rather than to an increased chance of hemolytic effectiveness of each.

The lysis of HEMPAS cells in acidified serum appears to be the result of activation of the classical C1-dependent pathway. Crookston et al. demonstrated an antibody present in normal serum which agglutinated HEMPAS cells in the cold; when the serum was acidified, it caused hemolysis at 37°C (7, 9, 10). The present experiments (Table VII) confirmed the observations of Crookston et al. that the antibody can be absorbed from serum by HEMPAS but not by normal or PNH cells. As shown by Table VIII, both C4 and C3 were bound to the membrane of HEMPAS but not PNH cells. In contrast, lysis of PNH cells in acidified normal serum appears to proceed solely by the alternate pathway (3, 4, 36). Lysis occurred equally well using adsorbed serum, and C4 was not fixed to the membrane during lysis (Table VII and VIII).

PNH red cells, like HEMPAS red cells, are lysed by small amounts of anti-I and complement, but the mechanism appears to be different. PNH cells bind the same amount of C4 as normal cells but bind three to seven times more C3 (36). This might be due to an increased number of "effective" $\overline{C42}$ sites, each capable of

fixing the normal number of C3 molecules. The $\overline{C42}$ complex is known to be unstable at 37°C since the C2 is lost from it (37). If the complex were more stable on PNH cells, then more C3 could be bound per C4 molecule. However, previous studies have shown that the rate of decay of the $\overline{C42}$ complex is the same on normal and PNH cells (2).

Alternatively, a normal number of effective $\overline{C42}$ sites may be present but each effective $\overline{C42}$ site is capable of fixing more C3 molecules. A third possibility is suggested by the studies of C3 fixation when the alternate pathway is activated. When PNH red cells are lysed by acidified normal serum, large quantities of C3 are bound to the lysed cells and little to the unlysed cells, suggesting a mechanism whereby bound C3 leads to the binding of more C3 (36). Such a mechanism is possible since C3b is one of the intermediates in the activation of the alternate pathway (38); thus, bound C3b might promote "local" activation of the alternate pathway. This appears to be exaggerated on PNH cells. Hence, the increased C3 binding seen when complement is activated by the classic pathway may be due in part to the binding of C3 through activation of the alternate pathway. Whatever the mechanism, it is clear that for a given degree of activation of the early steps of the classical pathway, more C3 is bound to PNH than to normal cells.

Further, more lysis occurs for a given amount of membrane-bound C3 on PNH cells compared with normal and HEMPAS cells (Fig. 6). The reason for this ability to establish "hemolytically active" sites is not known. Using activated $\overline{C42}$ in fluid phase, Götze and Müller-Eberhard found that three to five times as much C5 was bound to PNH cells as compared with normal cells, but that the amount of lysis obtained from each bound C5 was the same on PNH cells as on normal cells (5). However, recent data from our laboratory do not confirm these findings, and further effort is needed to determine the reasons for this increased proportion of hemolytically effective sites.

PNH cells but not HEMPAS red cells are lysed when the alternate pathway of complement is activated. This is probably because the increased fixation or efficiency of complement components occurs late in the sequence, but the early steps of the classical pathway, before the entry of the alternate pathway, for HEMPAS cells. Thus, activation of the alternate pathway, either in vitro or in vivo, will lead to the lysis of PNH but not HEMPAS cells. This probably accounts for the fact that the life span of the complement-sensitive PNH cells is markedly shortened (39) but that of HEMPAS cells is seldom so (7, 40).

The abnormal reactions of HEMPAS and PNH cells in immune lysis may not be unique to cells in those

disorders. The studies of Dacie and Lewis and their colleagues suggest that red cells in other blood diseases, many of which are characterized by dyserythropoiesis, are more susceptible than normal to immune lysis (11, 41, 42). The mechanism of the increased immune lysis in these conditions must be determined by tests such as those used in the present investigation.

ACKNOWLEDGMENTS

REFERENCES

1. Rosse, W. F., and J. V. Dacie. 1966. Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. I. The sensitivity of PNH red cells to lysis by complement and specific antibody. J. Clin. Invest. 45: 736.

2. Rosse, W. F., and J. V. Dacie. 1966. Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. II. The role of complement components in the increased sensitivity of PNH red cells to immune lysis. J. Clin. Invest. 45: 749.

3. Hinz, C. F., Jr., W. S. Jordan, Jr., and L. Pillemer. 1956. The properdin system and immunity. IV. The hemolysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria. J. Clin. Invest. 35: 453.

4. Götze, O., and H. J. Müller-Eberhard. 1972. Paroxysmal nocturnal hemoglobinuria: hemolysis initiated by the C3 activation system. N. Engl. J. Med. 286: 180.

5. Götze, O., and H. J. Müller-Eberhard. 1970. Lysis of erythrocytes by complement in the absence of antibody. J. Exp. Med. 132: 898.

6. Kabakçi, T., W. F. Rosse, and G. L. Logue. 1972. The lysis of paroxysmal nocturnal haemoglobinuria red cells by serum and cobra factor. Br. J. Haematol. 23: 693.

7. Crookston, J. H., M. C. Crookston, K. L. Burnie, W. H. Francombe, J. V. Dacie, J. A. Davis, and S. M. Lewis. 1969. Hereditary erythroblastic multinuclearity associated with a positive acidified-serum test: a type of congenital dyserythropoietic anaemia. Br. J. Haematol. 17: 11.

8. Heimpel, H., and F. Wendt. 1968. Congenital dyserythropoietic anemia with karyorrhexis and multinuclearity of erythroblasts. Helv. Med. Acta. 34: 103.

9. Crookston, J. H., and M. C. Crookston. 1972. Hereditary anemia with multinuclear erythroblasts ("HEMPAS"). Birth Defects Original Articles Series, Vol. VIII, no. 3. The Fourth Conference on The Clinical Delineation of Birth Defects, Part XIV. The National Foundation—March of Dimes. 15.

10. Crookston, J. H., M. C. Crookston, and W. F. Rosse. 1972. Red-cell abnormalities in HEMPAS (hereditary

erythroblastic multinuclearity with a positive acidified-serum test). Br. J. Haematol. 23 (Suppl.) : 83.

11. Lewis, S. M., P. Grammaticos, and J. V. Dacie. 1970. Lysis by anti-I in dyserythroipoietic anaemias; role of increased uptake of antibody. Br. J. Haematol. 18: 465.

12. Rosse, W. F. 1973. Variations in the red cells in paroxysmal nocturnal haemoglobinuria. Br. J. Haematol. 24: 327.

13. Hemlkamp, R. W., R. L. Goodland, W. F. Bale, I. L. Spar, and L. E. Mutschler. 1960. High specific activity iodination of γ-globulin with iodine-131 monochloride. Cancer Res. 20: 1495.

14. Mayer, M. M. 1961. Complement and Complement Fixation. In Experimental Immunochemistry. E. A. Kabat and M. M. Mayer, editors. Charles C Thomas, Publisher, Springfield, Ill. 2nd edition. 133.

15. Frank, M. M., H. J. Rapp, and T. Borsos. 1964. Studies on the terminal steps of immune hemolysis. I. Inhibition by trisodium ethylenediaminetetraacetate (EDTA). J. Immunol. 93: 409.

16. Rapp, H. J., and T. Borsos. 1970. The Molecular Basis of Complement Action. Appleton-Century-Crofts, New York.

17. Nilsson, U. R., and H. J. Müller-Eberhard. 1965. Isolation of B_{1F}-globulin from human serum and its characterization as the fifth component of complement. J. Exp. Med. 122: 277.

18. Logue, G. L., W. F. Rosse, and J. P. Gockerman. 1973. Measurement of the third component of complement bound to red cells in patients with cold agglutinin syndrome. J. Clin. Invest. 52: 493.

19. Hartmann, R. C., D. E. Jenkins, Jr., and A. B. Arnold. 1970. Diagnostic specificity of sucrose hemolysis test for paroxysmal nocturnal hemoglobinuria. Blood. 35: 462.

20. Dacie, J. V., and S. M. Lewis. 1968. Practical Haematology. Grune and Stratton, New York. 4th edition. 226.

21. Borsos, T., and H. J. Rapp. 1965. Hemolysin titration based on fixation of the activated first component of complement: evidence that one molecule of hemolysin suffices to sensitize an erythrocyte. J. Immunol. 95: 559.

22. Rosse, W. F., and J. B. Sherwood. 1970. Cold-reacting antibodies: Differences in the reaction of anti-I antibodies with adult and cord red blood cells. Blood. 36: 28.

23. Borsos, T., and H. J. Rapp. 1963. Chromatographic separation of the first component of complement and its assay on a molecular basis. J. Immunol. 91: 851.

24. Borsos, T., and E. J. Leonard. 1971. Detection of bound C3 by a new immunochemical method. J. Immunol. 107: 766.

25. Sitomer, G., R. M. Stroud, and M. M. Mayer. 1966. Reversible adsorption of C'2 by EAC'4: role of Mg^{2+}, enumeration of competent SAC'4, two-step nature of C'2a fixation and estimation of its efficiency. Immunochemistry. 3: 57.

26. Borsos, T., H. J. Rapp, and M. M. Mayer. 1961. Studies on the second component of complement. I. The reaction between EAC'1,4 and C'2: evidence on the single site mechanism of immune hemolysis and determination of C'2 on a molecular basis. J. Immunol. 87: 310.

27. Hoffman, L. G., and P. Meier. 1967. Statistical evaluation of reaction mechanisms in immune hemolysis. I. The dose response of the second component of complement. Immunochemistry. 4: 419.

28. Langmuir, I. 1918. The adsorption of gases on plane surfaces of glass, mica and platinum. *J. Am. Chem. Soc.* **40**: 1361.

29. Opferkuch, W., H. J. Rapp, H. R. Colten, and T. Borsos. 1971. Immune hemolysis and the functional properties of the second (C2) and fourth (C4) components of complement. II. Clustering of effective $C\overline{42}$ complexes at individual hemolytic sites. *J. Immunol.* **106**: 407.

30. Rosse, W. F. 1968. The fixation of the first component of complement (C'la) by human antibodies. *J. Clin. Invest.* **47**: 2430.

31. Evans, R. S., E. Turner, and M. Bingham. 1967. Chronic hemolytic anemia due to cold agglutinins: the mechanism of resistance of red cells to C' hemolysis by cold agglutinins. *J. Clin. Invest.* **46**: 1461.

32. Harboe, M., H. J. Müller-Eberhard, H. Fudenberg, M. J. Polley, and P. L. Mollison. 1963. Identification of the components of complement participating in the antiglobulin reaction. *Immunology.* **6**: 412.

33. Müller-Eberhard, H. J., A. P. Dalmasso, and M. A. Calcott. 1965. The reaction mechanism of B$_{1c}$-globulin (C'3) in immune hemolysis. *J. Exp. Med.* **123**: 33.

34. Ruddy, S., and Austen, K. F. 1969. C3 inactivator of man. I. Hemolytic measurement by the inactivation of cell-bound C3. *J. Immunol.* **102**: 533.

35. Ruddy, S., and Austen, K. F. 1971. C3b inactivator of man. II. Fragments produced by C3b inactivator

cleavage of cell-bound or fluid phase C3b. *J. Immunol.* **107**: 742.

36. Logue, G. L., W. F. Rosse, and J. P. Adams. 1973. Mechanisms of immune lysis of red blood cells in vitro. I. Paroxysmal nocturnal hemoglobinuria cells. *J. Clin. Invest.* **52**: 1129.

37. Borsos, T., H. J. Rapp, and M. M. Mayer. 1961. Studies on the second component of complement. II. The nature of the decay of EAC' 1,4,2. *J. Immunol.* **87**: 326.

38. Müller-Eberhard, H. J., and Götze, O. 1972. C3 proactivator convertase and its mode of action. *J. Exp. Med.* **135**: 1003.

39. Rosse, W. F. 1971. The life-span of complement-sensitive and -insensitive red cells in paroxysmal nocturnal hemaglobinuria. *Blood.* **37**: 556.

40. Verwilghen, R. L., S. M. Lewis, J. V. Dacie, J. H. Crookston, and M. Crookston. 1973. HEMPAS. Congenital dyserythropoietic anemia (Type II). *Q. J. Med.* **42**: 257.

41. Dacie, J. V., S. M. Lewis, and D. Tills. 1960. Comparative sensitivity of the erythrocytes in paroxysmal nocturnal haemoglobinuria to haemolysis by acidified normal serum and by high-titre cold antibody. *Br. J. Haematol.* **6**: 362.

42. Catovky, D., S. M. Lewis, and D. Sherman. 1971. Erythrocyte sensitivity to in vitro lysis in leukaemia. *Br. J. Haematol.* **21**: 541.