

Mechanisms of Immune Lysis of Red Blood Cells In Vitro

I. PAROXYSMAL NOCTURNAL HEMOGLOBINURIA CELLS

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ABSTRACT The effect of five different reactions which activate complement (antibody activation, reduction in ionic strength, acidification, cobra venom factor (CoF) activation, and inulin activation) upon normal and PNH cells was investigated, using normal serum and serum devoid of the fourth component of complement (C4) activity from patients with hereditary angioneurotic edema (HANE) as a source of complement.

Both normal and HANE serum lysed paroxysmal nocturnal hemoglobinuria (PNH) cells when complement was activated by acidification, CoF and inulin, indicating that the terminal steps of complement were activated in the absence of C4, presumably by the alternate or properdin pathway. Normal but not HANE serum lysed cells coated with anti-I, indicating that complement was activated by the C1-dependent classic pathway. HANE serum partially supported lysis by serum at reduced ionic strength, indicating that the activation of terminal complement components had occurred through both of the pathways of activation.

The amount of the third component of human complement (C3) which was bound to the membrane of lysed and unlysed cells by these procedures was determined by anti-C3 absorption and was found to differ for each method of complement activation. In general, more C3 was bound to lysed cells than to unlysed cells. For given conditions, more was bound to PNH cells than to normal cells. However, very much less bound C3 was required for lysis of the PNH cells than for lysis of normal cells. These two phenomena, especially the latter, account for the marked lysis of PNH cells when complement is activated.

Dr. Logue is a Research Associate of the Research and Education Branch of the Veterans Administration.

Dr. Rosse is the recipient of Research Career Development Award no. 5 KO4 CA-38862.

Received for publication 12 June 1972 and in revised form 18 December 1972.

Normal cells treated with AET (aminoethylisothiuronium bromide) did not bind more C3 than untreated cells and the lysed cells had less bound C3 than lysed PNH cells.

INTRODUCTION

A portion of the red cells (the complement-sensitive population) of patients with paroxysmal nocturnal hemoglobinuria (PNH)¹ are abnormal in that they are lysed more readily than normal cells in vitro by complement (1). At least five different procedures inducing the activation of complement have been used to detect these abnormal cells: acidification of normal serum (Ham test) (2), reduction in ionic strength of normal serum (sucrose lysis test) (3), activation of complement by specific antibody (4), activation of complement by a

¹ *Abbreviations used in this paper:* AET, aminoethylisothiuronium bromide; alternate pathway, the mechanism of activation of the terminal components of complement which does not require the activation of C1, C4, and C2 but which depends upon the activation of properdin and other proteins in serum, including C3 proactivator; Anti-C3, rabbit antibody to human C3; C1^{sp}, first component of guinea pig complement; C2^{sp}, second component of guinea pig complement; C4^{sp}, fourth component of guinea pig complement; C4^{hu}, fourth component of human complement; C3, third component of human complement; C3* Titer, the reciprocal of the dilution of human serum in EDTA which lyses 50% of a given concentration of E^{sh}AC142: this measures the combined function of the third through the ninth component; C1-dependent pathway, the mechanism of activation of the terminal components (C3 and C5-C9) which depends upon the activation of C1 and subsequent activation of C4 and C2; CoF, cobra venom factor; E^{hu}C3, human red cells coated with C3 (see text); E^{sh}C43^{hu}, sheep cells coated with human C4 and C3; E^{sh}AC142, sensitized sheep red cells with the first, fourth, and second components of guinea pig complement attached; HANE, hereditary angioneurotic edema; PHN, paroxysmal nocturnal hemoglobinuria; VBS, veranol-buffered saline; VBSucrose, isotonic sucrose buffer.

TABLE I
Complement Assays of Normal and HANE Serum

Serum	Incubation	C4 Titer (effective molecules)	C3* Titer (C3* H ₅₀)
Normal	None	7400	180
Normal	37°	6900	130
HANE	None	300	210
HANE	37°	<5	170

factor present in cobra venom (cobra venom lysis test) (5, 6), and activation of complement by inulin (7, 8).

Complement activation in these procedures may occur by two different pathways. In the C1-dependent pathway, antibody fixation effects the binding and activation of the first component of complement (C1) which then activates the fourth (C4) and second (C2) components. The combination of C4 and C2 (C4₂ complex) cleaves the third component (C3) to an active fragment which leads to the activation of the terminal complement components (C5–C9) (9).

Serum from patients with hereditary angioneurotic edema (HANE) lacks an inhibitor of the first component of complement (10). In this serum, C1 is readily activated in the fluid phase and activation of C4 and C2 occurs, both in vivo and upon incubation in vitro. Subsequent depletion of C4 and C2 occurs without the functional depletion of the later complement components, C3–C9 (11). Thus, HANE serum cannot support C1-dependent reactions.

C3 and the terminal components of the complement sequence may also be activated by an alternate mechanism which does not involve antibody or the C1-dependent reactions. This alternate pathway, first described by Pillemer et al. (12), may be activated by inulin, zymosan or other polysaccharides, and involves the complex interaction of several serum proteins which have not been well defined. This pathway also leads to cleavage of C3 and the activation of the terminal components of complement (13, 14). The third component of complement assumes a central role when complement is activated by either pathway, since it is the first step of the final common sequence leading to cell lysis.

C3 may be bound to the membrane of the red cell as a result of complement activation. Membrane-bound C3 may be detected by heterologous antibodies to native C3 (anti-C3). The amount of membrane-bound C3 may be measured by quantitating its interaction with anti-C3 (15, 16).

In the present investigation, we have studied the mechanisms of complement activation and lysis in the five in vitro procedures used to detect the abnormal cells of

PNH. Using HANE serum, we have determined whether the activation of the terminal steps of complement in each of these procedures occurs through the C1-dependent pathway or through the alternate (properdin) pathway. We have measured the amount of membrane-bound C3 engendered by these procedures and have related this to the degree of lysis and to the mechanism of activation of the terminal steps of complement.

METHODS

Buffers and complement reagents

Veranol-buffered saline (VBS), isotonic sucrose buffer (VBSucrose), 60% isotonic sucrose—40% VBS (60% sucrose), buffered 0.015 M ethylenediaminetetracetic acid, disodium salt (EDTA-VBS), and Alsever's solution were prepared as outlined in reference (9). Sheep red cells (E^{sh}), rabbit antibody to boiled sheep red cell stromata (anti-Forsman antibody), guinea pig serum as a source of complement, the C1 and C2 from guinea pig serum and sensitized E^{sh} with C4^{sp} on the surface (E^{sh}AC4) were prepared as previously described (9). Highly purified human C3 and rabbit anti-C3 were prepared as described in reference 15. Partially purified cobra factor (CoF) was obtained from Sephadex G-100 chromatography as previously described (5).

Human red cells and serum

Whole blood from normal donors and PNH patients was collected in an equal volume of Alsever's solution (17) and stored at 4°C until used. The red cells were washed thrice in VBS and resuspended in the appropriate buffer before use. Standard red cell suspensions containing 2.2×10^8 cells per cubic centimeter, prepared as described in reference 4, were used unless otherwise indicated.

Artificial PNH cells were prepared from normal cells by reaction with an 8% solution (wt/vol) of aminoethylisothiuronium bromide (AET) for 9 min (18). The complement lysis sensitivity of these cells, assayed by the method of Rosse and Dacie (4), was increased 7–10 times.

To obtain normal human serum as a source of complement, blood, drawn aseptically, was immediately cooled and centrifuged at 0°C. The plasma was removed and maintained at room temperature until a fibrin clot had formed. The serum was then frozen at –90°C until used. Type-compatible serum was used in all incubations with red cells.

Serum from patients with HANE was similarly obtained. Immediately before use, both HANE and normal serum were incubated at 37°C for 2 h in order to deplete the HANE serum of C4 without diminishing the concentration of the later components of the complement sequence. To determine the extent of inactivation of C4 in normal and HANE serum, assay for hemolytically active C4 was carried out as outlined in reference 19. The concentration of hemolytically-active C4 was calculated on a molecular basis, using the analysis of Rapp and Borsos (9). These assays were carried out in duplicate and the results are shown in Table I. The incubated HANE serum is functionally deficient in C4.

To demonstrate the functional presence of the later complement components, C3 and C5–C9, E^{sh}AC142 were reacted with dilutions of normal and HANE serum in EDTA-VBS, and lysis was determined (17). The results of this assay

of the terminal complement components, expressed as the reciprocal of the dilution of serum necessary to produce 50% lysis, are shown in Table I; both normal and HANE serum retained full activity of the later complement components (C3 and C5-C9).

In vitro diagnostic tests for PNH

Lysis by antibody and serum. Normal and PNH cells were lysed by anti-I antibody² (obtained from a patient with chronic cold agglutinin syndrome) and normal or HANE serum as the source of complement by incubating equal volumes of a standard suspension of red cells, anti-I in dilution providing a relative excess of antibody, and dilutions of serum (limited complement conditions) or with a concentration of serum providing a relative excess of complement and dilutions of antibody (limiting antibody conditions). The mixtures were incubated at 0°C for 15 min and at 37°C for 45 min.

Lysis by serum diluted in isotonic sucrose. Normal, PNH and AET-treated cells were lysed by serum diluted in a medium of reduced ionic strength (sucrose lysis test) by incubating 1 vol of 40% suspension of cells in 10 vol of normal and HANE serum diluted in VBSucrose at 37°C for 60 min.

Lysis by acidified serum. Normal, PNH and AET-treated cells were treated with acidified normal and HANE serum by mixing 1 vol of a 40% suspension of washed cells in 10 vol of neat or diluted serum which had been acidified to pH 6.4 with 0.15 M HCl. The mixture was incubated at 37°C for 60 min.

Inulin lysis test. Equal volumes of a standard suspension of normal and PNH cells, dilutions of a 2% suspension of inulin and serum were incubated at 37°C for 60 min.

Lysis by cobra venom factor. To test the lysis of PNH cells by cobra venom factor (CoF), equal volumes of a standard suspension of cells, partially purified CoF at a concentration of approximately 0.25 mg protein/milliliter and dilutions of normal and HANE serum were incubated together at 37°C for 60 min. To determine the fixation of C3 by CoF-activated serum, equal volumes of a standard suspension of cells in 0.015 M EDTA-VBS, dilutions of activated serum (made by incubating equal volumes of partially purified CoF and fresh normal serum), and fresh serum in 0.015 M EDTA-VBS were incubated at 37°C for 60 min.

Recovery of cells and stromata

After the in vitro lysis procedures, the amount of lysis was determined and the unlysed cells and the lysed cell stromata were recovered as follows: The incubation mixtures were centrifuged in a table-top centrifuge at 200 g for 2 min. The sedimented cells were resuspended in EDTA-VBS at 37°C and again centrifuged slowly at 200 g. After the second centrifugation, the sedimented cells were washed thrice in 37°C EDTA-VBS, twice in 37°C VBS, and were resuspended in standard concentration in 60% sucrose.

To recover the stromata of complement lysed cells, the supernatant fluid from the first and second slow centrifugations were recentrifuged at 37°C at 25,000 g for 15 min. The supernatant fluid after centrifugation was analyzed for free hemoglobin by measuring absorbance at 412 or 541 nm. The fraction of cells lysed in the initial incubation

² Kindly supplied by Dr. John H. Crookston. Toronto, Canada.

TABLE II
*Lysis of PNH Cells by Normal and HANE Serum
Which had been Incubated at 37°C for 2h*

Complement activation procedure	Serum dilution	Percent PNH cells lysed*	
		Normal serum	HANE serum
Antibody (Anti-I)	1/30	53.9	0
	1/5	88.7	1.2
Sucrose	1/20	77.1	42.6
Insulin	1/4	23.8	34.8
Cobra venom factor	1/4	48.7	58.6
Acidification	1/1	47.6	64.4

* 76% of the PNH red cells were complement sensitive (4).

mixture was determined by comparison with appropriate standards. Pellets of complement lysed stromata were washed once more at 37°C in EDTA-VBS and twice in VBS. The washed stromata were resuspended with vigorous vortex mixing in a volume of VBS calculated to give a suspension equivalent to 2.2×10^8 cells/milliliter.

When required, the cells surviving complement lysis were converted to stromata (water-lysed stromata) by suspending them in hypotonic 0.015 M EDTA, washing twice in VBS and resuspending to the original volume of the standard suspension which had been lysed. These procedures for recovery of red cell stromata have been previously shown to result in the quantitative recovery of the stromata, as measured by sialic acid determinations (20). The amount of C3 bound to the membrane of intact cells, water-lysed stromata and complement-lysed stromata was determined.

The measurement of membrane-bound C3

The quantity of C3 on the membranes of lysed and unlysed cells was determined by the anti-C3 absorption technique of Borsos and Leonard (16), as modified by Logue, Rosse, and Adams (21). Sheep cells coated with human C3 were prepared by incubating washed sheep cells in normal human serum for 20 min at 30°C, washing thrice with VBS, incubating for 20 min with 0.02 M 2-mercaptoethanol, washing thrice with VBS, and incubating with trypsin for 30 min at 37°C. The cells were washed and suspended to a concentration of 1×10^9 /milliliter. The quantity of anti-C3 required to lyse these cells maximally in the presence of guinea pig serum as a source of complement was determined by incubating 0.1 ml of the cells suspension and 0.1 ml of a dilution of rabbit antiserum to human C3 for 10 min at 30°C, then adding 0.2 ml of a 1/40 dilution of absorbed guinea pig serum, incubating for 1 h at 37°C and determining the percentage cells lysed. This amount of anti-C3 was incubated with known quantities of pure C3 for 1 h at 37°C and the amount of anti-C3 remaining was determined by its ability to lyse the E⁵⁴C43²⁰ as before. A calibration curve was drawn relating the decrease in lysis of the sheep cells to the amount of C3 which had been incubated with the anti-C3. Human cells and stromata were incubated with the standard concentration of anti-C3 and the amount of the anti-C3 removed was determined by the reduction in the lysis of the coated sheep cells as before. The amount of C3 on the membrane surface was determined with reference to the calibration curve, and was expressed as molecules of C3 bound per cell.

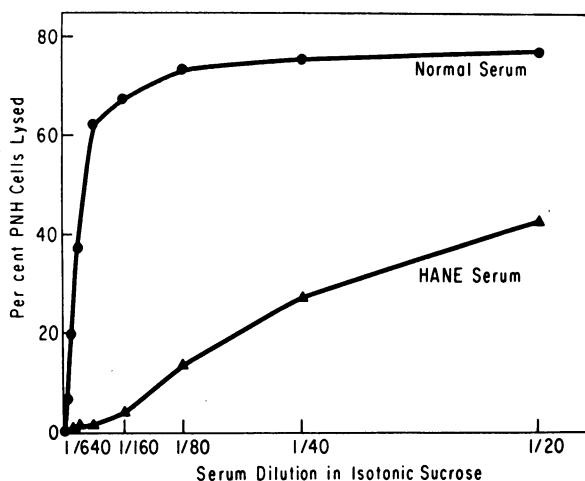


FIGURE 1 Lysis of PNH red cells by normal and HANE serum diluted in medium of reduced ionic strength. 76% of the PNH red cells were complement-sensitive by the method of Rosse and Dacie (4).

RESULTS

The results of lysis of PNH cells in normal and HANE serum when treated with antibody, reduced ionic strength, acidification, inulin, and CoF are shown in Table II. Normal cells were not lysed in any of these procedures at the concentrations of reactants used. A portion of the PNH cells were lysed in all procedures when normal serum was used. When HANE serum was acidified or treated with CoF or inulin, lysis of PNH cells was not diminished. HANE serum did not support lysis of PNH cells by anti-I. When HANE serum was diluted in medium of reduced ionic strength, the degree of lysis of PNH cells was less than that seen in normal serum, especially at higher dilutions of serum (see Fig. 1). Since HANE serum contains no C4, these findings suggest that C1-dependent complement reactions are not necessary for lysis of PNH cells by acidified serum, cobra, venom-treated serum, and inulin-treated serum, but that the C1-dependent pathway is essential to lysis by antibody and important for lysis by reduced ionic strength.

The binding of C3 to the lysed and to the unlysed cells. More C3 was detected on the membranes of lysed normal and PNH cells than on the membranes of unlysed cells, regardless of the mechanism of complement activation (Figs. 2-7). This did not appear to be an artifact of lysis since the same amount of antibody was detected whether the cells were intact or lysed by water, either before or after treatment with antibody and complement (Table III). When cells were lysed with activated C3 proactivator (cobra venom lysis) and complement, and the washed membranes were then treated with antibody and more complement, the same amount of C3 was de-

tected on the membranes as on the membranes of cells not lysed by activated C3 proactivator which were similarly treated with antibody and complement. Hence the increased C3 detectable on the membranes of lysed cells appears to be the result of the immune reaction and not due to an artifact of lysis or of the detection system.

When normal and PNH cells were lysed by antibody and complement, more C3 is attached to the membranes of the lysed PNH cells than to the membranes of the lysed normal cells at a given concentration of antibody (when it is limiting) or complement (when it is limiting). The difference is most marked when antibody is limiting and complement is in excess; three times as much C3 is fixed at low concentrations of antibody. The fixation of C3 to the unlysed PNH cells was greater than to unlysed normal cells when antibody was limiting, but was markedly less when complement was limiting; almost no C3 was attached to the unlysed PNH cells in these conditions (Fig. 4). Differences in C3 binding could not be attributed to differences in the adsorption of antibody since the anti-I used in these experiments (Step.) was adsorbed equally to normal and PNH cells.

Although more C3 is attached to lysed PNH cells than to lysed normal cells for a given amount of antibody or complement, less C3 is required for a given amount

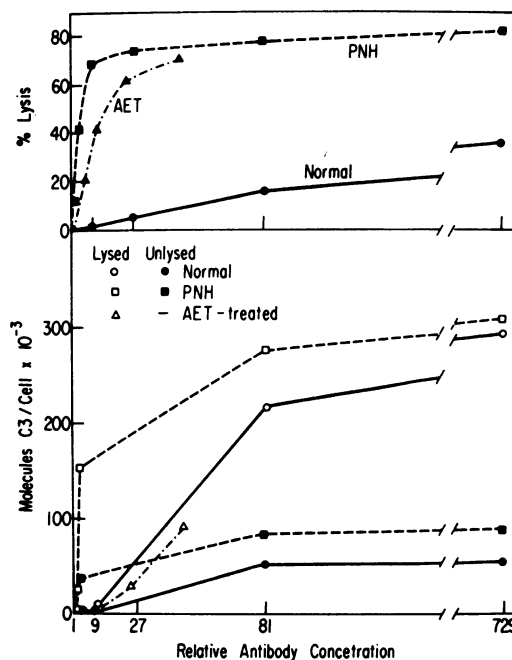


FIGURE 2 The fixation of C3 to the lysed and unlysed normal, AET-treated, and PNH cells by antibody when antibody concentration is limiting. The degree of lysis of the cells is shown in the upper panel on this and the following five figures. Relative antibody concentration of 1 is equivalent to a 1 in 48,600 dilution of antiserum, (anti-I [Step.]). 77% of the PNH cells were complement-sensitive.

of lysis of PNH cells in the range of partial lysis of the complement-sensitive cells (Fig. 8). About the same amount of C3 was bound to lysed normal cells for a given degree of lysis regardless of whether complement or antibody was limiting. However, less C3 was bound to the lysed PNH cells for a given degree of lysis when complement was limiting than when antibody was limiting.

The patterns of C3 binding to membrane seen when complement is activated by the alternate pathway, as by acidification of the serum, C3 proactivator activated by CoF, and inulin are somewhat more complex. Normal cells were not lysed in these tests and C3 was not bound to either normal cells or to the unlysed PNH cells in two of these tests (acidified serum lysis and activated C3 proactivator lysis). When the alternate pathway was activated by acidification of the serum, large amounts of C3 were bound to the lysed PNH cells but very much less was bound when this pathway was activated by cobra venom. When the alternate pathway was activated by inulin, C3 was bound to the membranes of the lysed and unlysed PNH cells and to normal cells in about equal quantities.

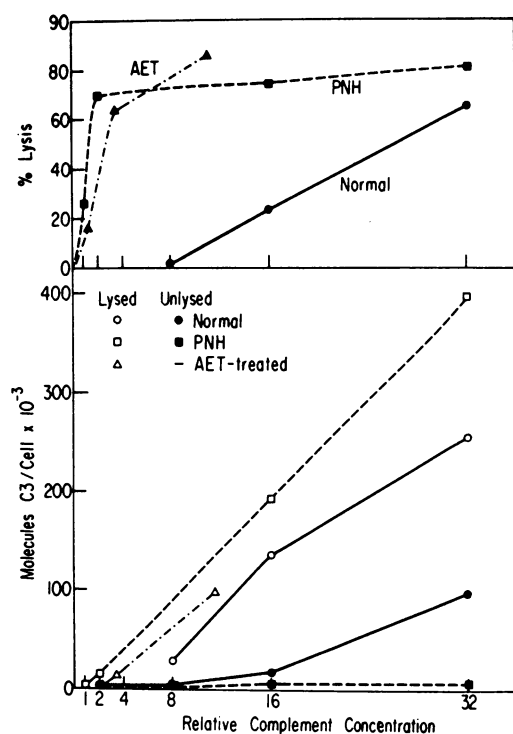


FIGURE 3 The fixation of C3 to the lysed and unlysed normal, AET-treated, and PNH cells by anti-I (Step.) and serum when complement is limiting. Relative complement concentration of 1 is equivalent to a 1 in 64 dilution of normal serum. 77% of the PNH cells were complement-sensitive.

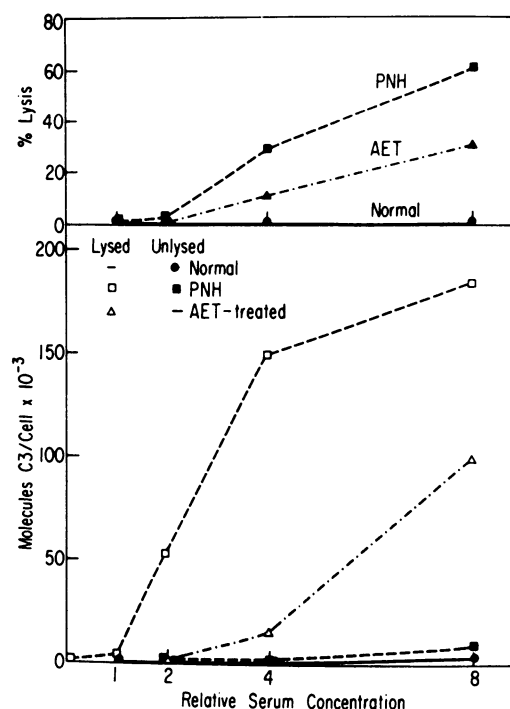


FIGURE 4 The fixation of C3 to lysed and unlysed normal, AET-treated and PNH cells by acidified normal serum. Relative serum concentration of 1 is equivalent to a 1 in 8 dilution of normal serum. 75% of the PNH cells were complement-sensitive.

The amount of C3 bound per lysed PNH cell at different levels of partial lysis of the complement-sensitive PNH cells by the six different reactions is shown in Fig. 9. For given degree of lysis, very large amounts of C3 were bound when complement was activated by acidification of the serum. Decreasing amounts were fixed by antibody lysis (antibody limiting), antibody lysis (complement limiting), inulin lysis, activated C3 proactivator lysis, and sucrose lysis.

The lysis of AET-treated cells by antibody and acidified serum demonstrated that these cells bound less C3 than PNH cells, and were lysed by very small amounts of bound C3 (Figs. 2, 3, 4, and 8).

DISCUSSION

PNH cells were used in early studies of the mechanisms of hemolysis of human red cells by complement (2, 22). However, it became apparent that the lysis of PNH cells in normal serum in vitro or in vivo could not be explained by activation of C1 by antibody, since specific antibodies directed against PNH cells could not be demonstrated by any technique (2, 23, 24). Pillemer and his co-workers (12), using PNH cells, proposed an alternate pathway for the activation of complement by a group of proteins, one of which was properdin (22). The more recent in-

TABLE III
The Detection of C3 on Intact and Lysed Cells

Reaction	State of cells during reaction	State of cells during analysis	Cells analyzed	Molecules of C3 per cell
Acidified serum lysis	Intact	Water-lysed	All	68,400
	Water-lysed	Water-lysed	All	68,400
	Lysed with CoF and serum	Water-lysed	All	60,700
Anti-I (1/1600)	Intact	Water-lysed	All	48,300
	Water-lysed	Water-lysed	All	33,300
	Lysed with CoF and serum	Water-lysed	All	24,800
Anti-I (1/800)	Intact	Intact	Survivors*	167,200
	Intact	Water-lysed	Survivors*	110,200
	(1/3200)	Intact	Survivors*	19,500
	Intact	Water-lysed	Survivors*	17,500

* Only those cells surviving the original immune lytic reaction were analyzed.

vestigations of Götze and Müller-Eberhard (6, 7) and Kabakci, Rosse, and Logue (5) have confirmed that activation of this alternate pathway may lead to the lysis of PNH cells. In the present experiments, we have further defined which of these pathways is activated in several in vitro procedures which are known to lyse PNH cells.

When complement is activated by antibody, the C1-dependent steps are necessary; for this reason, anti-I

does not lyse PNH cells when serum from patients with HANE is used as a source of complement since this serum lacks components which are necessary for the C1-dependent reactions. The C1-dependent steps also appear to be important when complement is activated by reduction in ionic strength. Reduced ionic strength binds IgG immunoglobulin molecules nonspecifically to the cell membrane and these in turn activate C1 (25). Reduction in ionic strength also appears to activate the alternate pathway of complement activation, since HANE

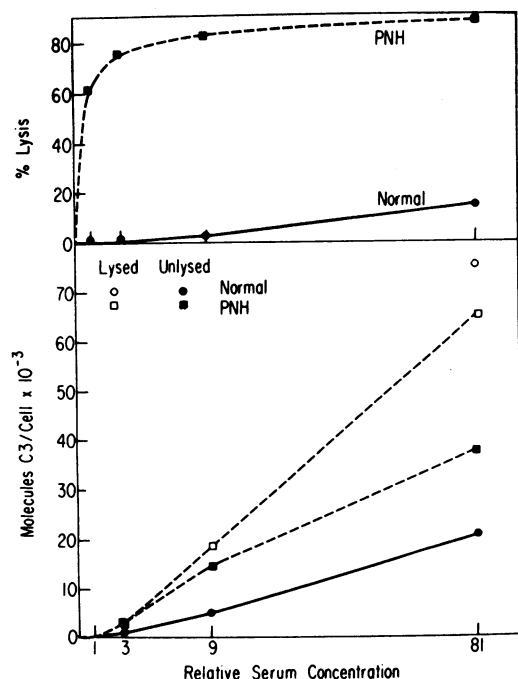


FIGURE 5 The fixation of C3 to lysed and unlysed normal and PNH cells by reduction in ionic strength. Relative serum concentration of 1 is equivalent to a 1 in 405 dilution of normal serum. 75% of the PNH cells were complement-sensitive.

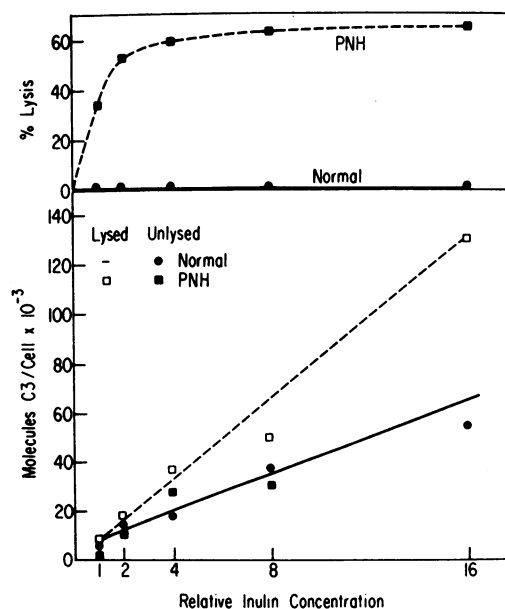


FIGURE 6 The fixation of C3 to lysed and unlysed normal and PNH cells by inulin and serum. A relative concentration of inulin of 1 is equivalent to a 1 in 16 dilution of a 2% inulin suspension. 60% of the PNH cells were complement-sensitive.

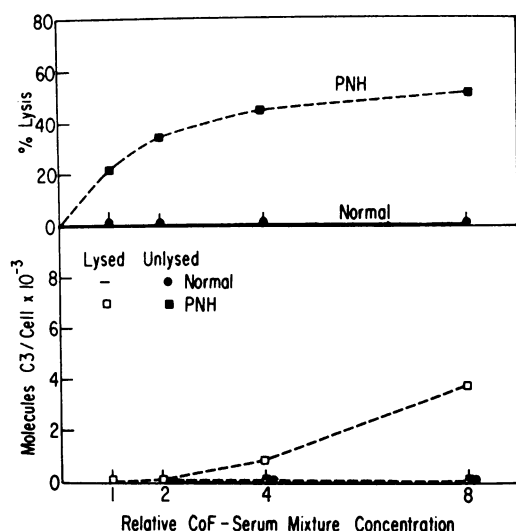


FIGURE 7 The fixation of C3 to lysed and unlysed normal and PNH cells by activated proactivator (CoF-activated serum) and fresh serum. Relative concentration of the CoF-serum of 1 is equivalent to a 1 in 64 dilution of the mixture (activated proactivator). 60% of the PNH cells were complement-sensitive.

serum in high concentration is capable of lysing PNH cells.

The other three reactions which lyse PNH cells by activation of complement (inulin activation, CoF activation, and acidification) do not require the C1-dependent reactions, since the amount of lysis obtained with HANE serum is the same or greater than that obtained with normal serum. This confirms that the activation of the late components of complement occurs in each case through the alternate pathway (6, 7).

When either normal or PNH cells are lysed by complement, more C3 is detectable on the lysed cells than on the unlysed cells. This difference is most striking when lysis is obtained by acidified serum and is least when complement is activated by inulin. The increased C3 detected on lysed cells does not appear to be due to an increased detectability since the same amount of C3 is detected on cells surviving antibody-complement lysis before and after osmotic lysis. This increase in bound C3 does not appear to be the result of lysis per se since when cells are lysed by other means (osmotic stress, previous lysis by complement) before treatment with activated complement, the amount of C3 bound is not greater than that on intact cells treated in the same way.

This difference between lysed and unlysed cells might be explained by postulating that cells were not equally coated with C3 and that the more heavily coated cells were lysed. This would predicate a very wide disparity in the amount of complement fixed by different cells, especially when complement is activated by acidification

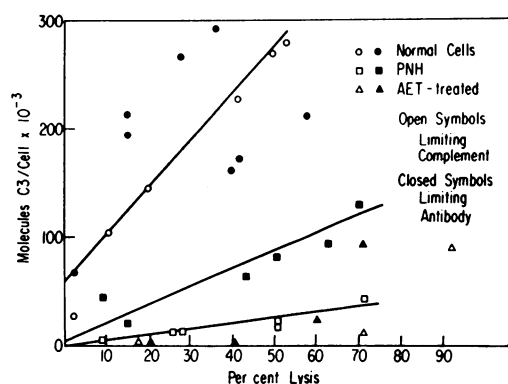


FIGURE 8 The relationship between the degree of lysis and the amount of C3 bound to the lysed normal, PNH or AET-treated cells when complement is activated by antibody. The results of experiments where antibody was present in excess and complement concentration was limiting are shown in open symbols, those in which antibody concentration was limiting and complement was present in relative excess, in closed symbols. For PNH cells, only results of experiments in which the complement-sensitive population was partially, but not completely, lysed are shown.

of the serum where no C3 was fixed to unlysed cells and marked amounts were fixed to the lysed cells. If this hypothesis is true, then very small amounts of bound C3 are sufficient to lyse these cells since all cells with detectable membrane-bound C3 are lysed. This would suggest that much of the bound C3 on the lysed cells by acidified serum was epiphenomenal "over-kill," and not

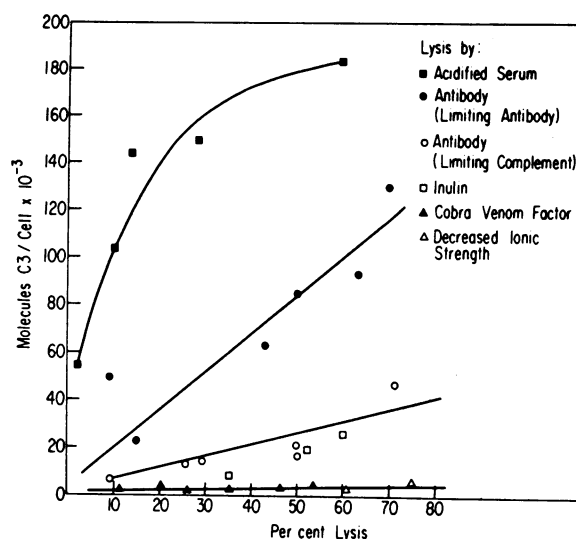


FIGURE 9 The relationship between the degree of lysis of PNH cells and the quantity of C3 bound to the lysed cells by different mechanisms of activating complement. The results are from experiments in which the complement-sensitive population was partially, but not completely, lysed.

essential to the lysis of the cells. The genesis of such large amounts of bound C3 by fluid-phase reactions is enigmatic.

In most instances, PNH cells bind more C3 than normal cells under the same conditions. This is especially true when complement is activated by limiting amounts of antibody. This increased C3 binding could not be attributed to increased binding of antibody since the anti-I used in these experiments is bound equally to normal and PNH cells. This increased binding of C3 may result from the membrane defect of PNH and may play a role in the remarkable susceptibility of these cells to lysis by complement.

The major effect of the membrane defect in PNH is, however, the remarkable susceptibility of these cells to lysis when small amounts of C3 are bound. When complement is activated by the alternate pathway or by reduced ionic strength, very small amounts of membrane-bound C3 appear to be necessary for lysis, even though, as pointed out above, large amounts may be bound. It is possible that the binding of C3 may be by-passed in these reactions since Lachmann and Thompson (26) have shown that fluid-phase activated C5, C6, and C7 might be responsible for passive ("reactive") lysis of cells.

When complement is activated by antibody, relatively larger amounts of C3 are required for lysis than when it is activated by the alternate pathway. This suggests that antibody-organized complement reactions are relatively less efficient in bringing about lysis than fluid-phase, alternate-pathway initiated reactions. Nevertheless, the amount of membrane-bound C3 required for lysis of PNH cells was clearly less than that required for lysis of normal cells.

AET-treated cells appear to share these membrane abnormalities with PNH. These cells are lysed as efficiently as PNH cells by very small amounts of membrane-bound C3 but fix lesser amounts of C3 than PNH cells for the same concentration of antibody or complement. The latter effect may account for the fact that these cells are not as sensitive to antibody-mediated complement lysis as PNH cells.

The increased sensitivity of PNH and AET-treated cells to the lytic action of complement may be due to several mechanisms: (a) one or more of the later components of complement may be attached in greater amount to these cells than to normal cells, (b) one or more of the later components of complement may be more effective in propagating the sequence or in effecting the ultimate lytic lesion, or, (c) both of these processes may occur. Götze and Müller-Eberhard (6) have adduced evidence that more C5 is attached to PNH cells than to normal cells, but each attached C5 is no more effective in causing lysis of PNH cells than of normal cells. The

mechanism and the biochemical background for the increased lysis of PNH cells remains enigmatic.

ACKNOWLEDGMENTS

This project was supported by National Institutes of Health Grant no. 5 R01-CA-10267.

The patients were hospitalized on the Clinical Research Unit under Grant RR30 from the General Clinical Research Center of the National Institutes of Health.

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