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**Research Article**



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# The Initiation and Enhancement of Human Red Cell Lysis by Activators of the First Component of Complement and by First Component Esterase; Studies Using Normal Red Cells and Red Cells from Patients with Paroxysmal Nocturnal Hemoglobinuria *

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Recent studies from this laboratory have described the anti-C' [1] properties of poly I (1, 2). Inquiry into the mechanism by which poly I inhibits C'-dependent immune hemolysis has revealed that this material interacts with the C'1q (11 S) (3) portion of the C'1 molecule, thereby preventing the attachment of C'1 to the sensitized red cell (4). Addition of poly I to C' also results in inactivation of C'4. The latter effect is mediated via activation of the C'1 molecule and the conversion of C'1 proesterase (C'1s) to the active esterase form (5). This conclusion, although based on indirect evidence, is strengthened by the observation that poly I shares with C'1a and purified C'1 esterase the ability to evoke vascular permeability in guinea pig skin (6, 7). On the basis of this evidence an analogy has been drawn between the effects of poly I on the C' system, and those of antigen-antibody complexes or heat aggregated gamma globulin (5, 6).

After having thus defined in detail the mechanism of poly I inhibition of immune red cell lysis, attention was turned to the effects of this material on the hemolysis *in vitro* of red cells from patients afflicted with PNH. There can be little doubt that the C' system participates in PNHE lysis, since removal or inactivation of any one of the four major components of C' will destroy the capacity of human serum to support PNHE lysis (8). It was naturally anticipated that poly I, being an anti-C' substance, would inhibit the PNHE hemolytic system. In fact, it was found that the addition of poly I to human serum provoked substantial stimulation of PNHE hemolysis. In addition it was found that the addition of poly I to human serum could, under appropriate conditions, induce substantial *in vitro* hemolysis of normal human erythrocytes (NHE). Other activators of C'1, as well as C'1a and C'1 esterase, behaved in a similar manner. This paper describes these hemolytic systems with respect to kinetics, pH optima, cat-

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[1] The following abbreviations are used: C', complement; anti-C', anticomplementary; $C'H_{50}$, 50% hemolytic unit of complement; C'1, C'2, C'3, C'4, the first, second, third, or fourth component of complement; R1, R2, R3, R4, RP, serum lacking the designated complement component or properdin; C'1a, C'2a, activated first or second complement components; C'1q, C'1s, subcomponents of the first component of complement; C'3a, C'3b, C'3c, C'3d, four factors that together comprise total third component activity; E, erythrocytes; S, antigen site on the red cell membrane; A, antibody or amboceptor; EA, sensitized erythrocytes (usually sheep red cells); EAC'. . . , sensitized erythrocytes bearing complement components as designated by numerical subcripts; E*, red cells irreversibly damaged by the action of complement; S*, red cell membrane site damaged by complement; C'1EI, the serum inhibitor of first component esterase; PNH, paroxysmal nocturnal hemoglobinuria; PNHE, red cells from patients with paroxysmal nocturnal hemoglobinuria; NHE, normal human red cells; poly I, poly A, poly U, poly C, biosynthetic homopolymers of inosinic, adenylic, uridylic, and cytidylic acids; poly (I + A), poly (I + C), double stranded hydrogen bonded helices composed of equimolar amounts of polyinosinic and polyadenylic acids or polyinosinic and polycytidylic acids; EDTA, ethylenediamine tetraacetate.

ion requirements, and requirements for the participation of C' components; offers a theory on the mechanism of the induction of a C'-dependent hemolytic system by "anticomplementary" substances via "indifferent" activation of fluid phase C' components; and comments upon the possible significance of these phenomena in relationship to acquired hemolytic anemia in humans.

## Methods

*Polynucleotides.* The methods of preparation or isolation of the polynucleotides have been described in detail (1). Polynucleotides were dissolved in unbuffered 0.15 M NaCl at a concentration of 10 μmoles P per ml and were stored frozen at −20° C.

*Human serum reagents.* Five hundred ml blood was removed from healthy donors in the postprandial state and stored without anticoagulant at 4° C overnight. The following morning the clotted blood was centrifuged, and the serum was collected, pooled according to ABO blood type, and frozen in 6- to 10-ml portions at −85° C in a mechanical freezer. Preliminary experiments involving both NHE and PNHE showed that the stimulation of *in vitro* hemolysis by poly I occurred without the participation of any antibodies directed against the red cell, i.e., that hemolysis could be provoked by poly I even if red cells and serum were from the same donor. Accordingly, serum and red cells were used without regard to major or minor red cell antigens, provided only that the cell-serum combination was compatible by the usual cross-matching techniques. It was convenient to utilize AB $Rh^+$ donors as the serum source, and therefore, after preliminary studies had defined the nature and reproducibility of the phenomena under study, large pools of serum from AB $Rh^+$ donors were collected and frozen for future use.

Serum deficient in the various components of C' (9) and RP (10) were prepared by published procedures. If not used immediately after preparation, these serum reagents were also stored at −85° C. Heat inactivated serum was prepared by incubating serum at 56° C for 30 minutes.

Serum containing EDTA salts was prepared as follows: $Na_2MgEDTA$ or $Na_2HEDTA_2$ was dissolved in water, titrated to the desired pH (6.5 or 7.4), and diluted to a concentration of 0.15 M that served as a stock solution. Sufficient EDTA solution was added to serum to obtain the desired EDTA concentration (*ca.* $2 \times 10^{-2}$ M), and after standing at room temperature for 30 minutes the serum was employed as indicated. Control serum was diluted with an equivalent volume of 0.15 M NaCl.

*Human red cells.* Blood from both normal and PNH donors was drawn under aseptic conditions into either one-sixth volume ACD solution[3] or into an equal volume of Alsevers solution, and was left at 4° C. Most studies were done on cells that were less than 7 days old, but since the length of storage seemed to have little effect upon the susceptibility of the red cells to poly I-induced hemolysis (*vide infra*), cells were occasionally kept and used for up to 14 days. Before use the red cells were washed 3 times in 0.15 M NaCl, and after the buffy coat had been carefully aspirated, they were suspended to 20% by volume in 0.15 M NaCl.

*Other materials*

A. *Gamma globulin aggregates.* Soluble, heat aggregated, gamma globulin was prepared according to the method of Hinz and Mollner (11) with commercially obtained human 7 S gamma globulin as the starting material.[4] The stock solution of heat aggregated gamma globulin in 0.15 M NaCl contained 43.0 mg protein per ml.

B. *Antigen-antibody aggregates.* Antiserum to bovine serum albumin was prepared in rabbits, cleared by high speed centrifugation, heated at 56° for 30 minutes, and diluted 1:2 with 0.15 M NaCl containing $1.2 \times 10^{-2}$ M $Na_3HEDTA$. This antiserum dilution was then mixed with an equal volume of bovine serum albumin solution containing 370 μg protein per ml (an amount of antigen designed to yield an antigen-antibody precipitate formed at equivalence according to pretitration). The mixture was kept at 4° C for 48 hours, and the precipitate was then isolated by centrifugation, washed 3 times in 0.15 M NaCl, and finally suspended in saline at a protein concentration of 30.9 mg per ml.

C. *Streptokinase.*[5] This enzyme was dissolved in 0.15 M NaCl at a concentration of 10,000 μ per ml and was stored at −85° C.

D. *Dextran sulfate.*[6] Two dextran sulfates of mean molecular weights 60,000 (60 S) and 200,000 (200 S) were dissolved in 0.15 M NaCl at a concentration of 5,000 μg per ml and kept at 4° C.

E. *Partially purified C'1a.* Two separate preparations of human C'1 were prepared by the method of Lepow, Ratnoff, Rosen, and Pillemer (12). Before use the material was activated as described by the same authors. The activated preparations contained 48 and 37.6 U C'1 esterase per ml (13).

F. *C'1 esterase.* C'1 esterase was isolated by DEAE ion exchange resin chromatography of a human serum euglobulin fraction as outlined by Haines and Lepow (14). Only the first resin passage was performed. The fractions containing esterase activity were pooled, dialyzed against ion free water, and dissolved in 0.15 M NaCl. The final preparation, containing 352 U C'1 esterase and 2 mg protein per ml, was stored at −85° C.

G. *C'1 esterase serum inhibitor.* This material, iso-

[2] Geigy Industrial Chemicals, Ardsley, N. Y.

[3] Vacutainers, Becton Dickinson and Co., Columbus, Neb.

[4] Pentex Laboratories, Kankakee, Ill.

[5] Varidase, Lederle Laboratories, Pearl River, N. Y.

[6] Sigma Chemical Co., St. Louis, Mo.

lated by the method of Pensky, Levy, and Lepow (15), was dissolved in 0.15 M NaCl. The stock solution, stored frozen at −85° C, contained 11.7 U C'1 EI per ml (13).

*Estimation of in vitro hemolysis.* The technique used in estimating red cell hemolysis was similar to that described in a previous publication on pH-dependent hemolytic systems (16). The basic system consisted of 1 ml human serum or human serum reagent, a precisely measured volume of test substance dissolved in unbuffered 0.15 M NaCl (usually 0.1 ml)[7] or an equivalent volume of saline only as a control, and 0.05 ml of a 20% suspension of erythrocytes. If the pH had been lowered from that of the native thawed serum, the serum was kept under oil until completion of the experiment. All reagents were prewarmed to 37° C before mixing, and hemolysis was carried out at the same temperature for 30 minutes with occasional gentle agitation to keep the cells evenly suspended. The tubes were then centrifuged, and the supernatant fluid was removed with a Pasteur pipette. The optical density of the supernatant fluid at 540 mμ was measured against an appropriate serum blank in matched silica cuvettes with a 1-cm light path. The per cent hemolysis was determined by comparison with a cell-serum sample that had been completely hemolyzed by freezing and thawing. The 100% OD at 540 mμ of 0.05 ml cell suspension in the usual test volume (1.15 ml) ranged from 1.3 to 2.1; the lower values were observed with PNHE, presumably because of their lower mean corpuscular hemoglobin concentration (17).

The pH of serum immediately after thawing was generally 7.5 to 7.7. Serum was acidified directly before use by titration with small amounts of 0.3 N HCl and was kept under oil at 4° C until needed. All pH values refer to measurements taken before incubation at 37° C; it is recognized that pH values increase by approximately 0.5 pH U after 30 minutes incubation at 37° C (18).

During the initial phases of this study variable results were obtained when attempts were made to reproducibly increase or initiate hemolysis by poly I addition to serum. This variability revealed the critical importance of the sequence and timing of the various additions to the hemolytic system. For reproducible results it is imperative that the addition of erythrocytes and test material be, for all practical purposes, simultaneous; the ability of test material to initiate or provoke hemolysis diminishes in minutes if test material and serum are preincubated at 37° C before the addition of cells. In addition (in the case of PNHE hemolysis) with the further passage of time such a test material-serum mixture gradually loses hemolytic capacity (*vide infra*). Since actual simultaneous addition was not practical, preaddition of cells, mixing, and subsequent addition of the other constituent was employed. This procedure took less than 15 seconds; it was considered, in the time lapse experiments, to be the "zero" time sample. Unless otherwise noted all results described are derived from this modified technique. All experiments were performed at least twice; all data reported have been verified as reproducible.

In addition to the importance of the time and sequence of addition of test materials in these hemolytic systems, dilution of serum was found to be critical. Thus, in experiments with the R reagents, where such reagents as R1 and R4 were appreciably diluted with respect to the original serum (dilutions of 1:1.25 and 1:1.45, respectively), it was imperative to include appropriate saline diluted control serum tests in order to assess fairly the effect of the specific C' component depletion upon the hemolytic systems. The point will be more fully discussed in the following section.

Protein determinations were done by a modified Folin technique (19). A Beckman Zerostatic pH meter and Zeiss PMQ II spectrophotometer were used throughout.

## Results

Table I shows the effects of the addition of 1.0 μmole P poly I to the hemolytic system using red cells from 10 PNH patients and 12 normal individuals. NHE undergo less than 10% hemolysis with poly I addition at pH 7.6; PNHE, on the other hand, consistently show > 30% hemolysis at this pH in the presence of poly I, even if, in the pH 7.6 control, they display little or no lytic susceptibility. At pH 6.5 all cells show substantial hemolysis in the presence of poly I. PNHE, which invariably hemolyze appreciably at this pH, are hemolyzed 2 to 3 times more effectively as a result of poly I addition. NHE, which in the absence of poly I are incapable of hemolysis even at pH 6.5, in its presence display levels of hemolytic susceptibility comparable to those of PNHE in the pH 6.5 saline controls. Hemolytic tests using the red cells of a large variety of hospital patients with and without hematologic disease have failed to detect any individual whose cells are resistant to hemolysis at pH 6.5

[7] Upon the addition of > 0.5 μmole poly I to 1 ml whole human serum (pH 6.5) an immediate haze appeared. Preliminary study of the easily centrifuged precipitate revealed the presence of poly I and protein. A similar haze was not provoked by other polynucleotides, nor by poly (I + A) or poly (I + C), nor would poly I evoke precipitate formation in heated serum or R1. Precipitate formation has been observed in R2 and in $Na_3$HEDTA chelated serum. The nature of the proteins precipitated by poly I is presently under investigation; gamma globulin has been identified as being present in the precipitate. A similar precipitate is formed with the addition of > 125 μg dextran solution to 1 ml serum. Since the precipitate was easily brought down during centrifugation, it did not interfere with optical density determinations on the supernatant during hemolysis experiments.

TABLE I

*Effect of poly I on hemolysis of PNH and normal erythrocytes**

| Patient | PNH | | | | Patient | Normal | | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH 7.6 | | pH 6.5 | | | pH 7.6 | | pH 6.5 | |
| | Saline control | Poly I | Saline control | Poly I | | Saline control | Poly I | Saline control | Poly I |
| | % lysis | | % lysis | | | % lysis | | % lysis | |
| 1 | 5 | 31 | 22 | 66 | 1 | 1 | 5 | 1 | 48 |
| 2 | 8 | 61 | 36 | 92 | 2 | 1 | 0 | 1 | 28 |
| 3 | 5 | 49 | 30 | 80 | 3 | 1 | 5 | 1 | 42 |
| 4 | 11 | 61 | 53 | 82 | 4 | 0 | 1 | 1 | 37 |
| 5 | 25 | 57 | 43 | 76 | 5 | 2 | 4 | 1 | 43 |
| 6 | 2 | 64 | 41 | 84 | 6 | 1 | 2 | 1 | 27 |
| 7 | 9 | 59 | 37 | 63 | 7 | 1 | 3 | 3 | 59 |
| 8 | 9 | 78 | 47 | 95 | 8 | 2 | 2 | 2 | 38 |
| 9 | 8 | 43 | 20 | 66 | 9 | 2 | 2 | 3 | 40 |
| 10 | 11 | 71 | 32 | 86 | 10 | 1 | 2 | 2 | 36 |
| | | | | | 11 | 1 | 2 | 3 | 46 |
| | | | | | 12 | 1 | 1 | 2 | 36 |
| Range | 2–25 | 31–78 | 20–53 | 63–95 | | 0–2 | 0–5 | 1–3 | 27–59 |

* PNH = paroxysmal nocturnal hemoglobinuria.

in the presence of poly I. Although there is little or no overlap in the range of poly I-induced hemolysis at pH 6.5 when NHE (27 to 59%) and PNHE (63 to 95%) are compared, an occasional test with NHE under these conditions has resulted in > 60% hemolysis (for example, see Table V). From these data it is apparent that the poly I-induced hemolytic system will invariably distinguish between PNHE and NHE at pH 7.6.

*Other polynucleotides.* The following naturally occurring and synthetic polynucleotides were studied for their ability to induce NHE hemolysis or to provoke PNHE hemolysis: RNA derived from rat liver, guinea pig liver, and mouse spleen; poly A, poly U, poly C; and apurinic acid derived from calf thymus DNA. One μmole P of these materials fails to induce or provoke an increase in NHE or PNHE hemolysis at either pH 6.5 or 7.6.

*Effect of hydrogen bonded helix formation upon the capacity of poly I to provoke hemolysis.* Poly (I + C) and poly (I + A) are incapable of stimulating PNHE hemolysis. In addition, poly I incorporated into such helices is incapable of inducing NHE hemolysis.

*Effect of increments of poly I upon poly I-induced hemolysis.* Figure 1 illustrates the ability of varying amounts of poly I to stimulate hemolysis of NHE and PNHE. Maximal enhancement of PNHE hemolysis is obtained with addition of 0.6 μmole P poly I at both pH 7.6 and pH 6.5. Eight-tenths μmole P poly I yields maximal NHE hemolysis in acidified serum. With larger amounts of poly I there is a slight but definite reduction in hemolysis in all instances. Despite this, and largely in an attempt to keep experimental conditions consistent with those in use before these observations, we continued to use 1 μmole P poly I as the standard test dose in most of the experiments.

*Other test materials.* Dextran sulfate, C'1a, C'1

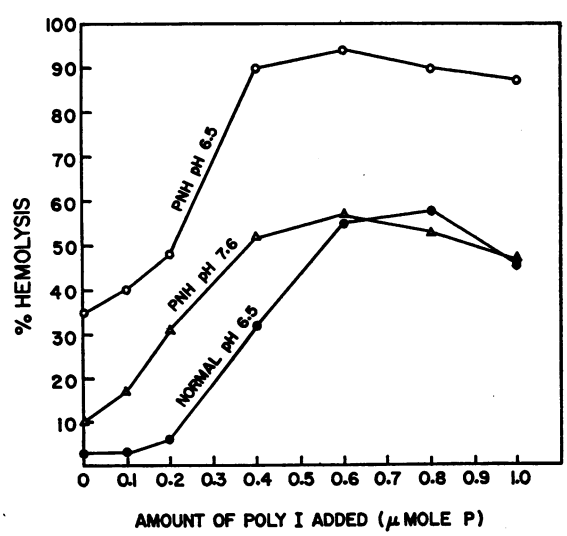


FIG. 1. ABILITY OF INCREMENTS OF POLY I TO INDUCE OR ENHANCE HEMOLYSIS.

esterase, streptokinase, and aggregated γ-globulin all share with poly I the capacity to enhance hemolysis of PNHE, although none is as efficient as poly I (Table II). The dose dependency of this effect, with large doses of test material being inhibitory, is strikingly illustrated by dextran sulfate, C'1 esterase, and aggregated γ-globulin. Dextran sulfate is the only other test material capable of provoking NHE lysis, and here too its effect is less pronounced than that of the polynucleotide.

*Effect of pH upon test material induced hemolysis.* As noted in Table I, poly I-induced hemolysis proceeds more effectively at pH 6.5 than at pH 7.6. Figure 2A illustrates more completely the effects of variations in pH upon the ability of both PNHE and NHE to hemolyze in the presence and absence of poly I. NHE are resistant to hemolysis at all pH's tested; in the presence of poly I, optimal hemolysis of NHE occurs at pH 6.5 to 6.8. PNHE, as expected, display maximal hemolysis at pH 6.2 to 6.5, and poly I enhancement results in maximal total hemolysis also at pH 6.5. The greatest relative increase in PNHE hemolysis with poly I addition occurs at pH 7.6, when hemolysis is increased to six times the control level. Similar results were obtained with dextran sulfate (Figure 2A) and in the case of PNHE with

TABLE II

*Ability of increments of various test materials to induce or enhance human red cell lysis*

| C'1a PNH cells Esterase units added (in 0.4 ml saline) | Case PE | | | | Case ST | | | |
|---|---|---|---|---|---|---|---|---|
| | pH 6.5 | | pH 7.6 | | pH 6.5 | | pH 7.6 | |
| | OD 540 | % lysis | OD 540 | % lysis | OD 540 | % lysis | OD 540 | % lysis |
| 9.6 | 1.169 | 52.5 | 0.070 | 3.1 | 1.020 | 48 | 0.115 | 5.4 |
| 4.8 | 1.034 | 46.5 | 0.154 | 6.9 | 0.939 | 44 | 0.203 | 9.6 |
| 2.4 | 1.009 | 45 | 0.185 | 8.3 | 0.940 | 44 | 0.152 | 7.2 |
| 1.2 | 0.860 | 39 | 0.137 | 6.1 | 0.731 | 34.5 | 0.133 | 6.3 |
| 0.6 | 0.805 | 36 | 0.162 | 7.3 | 0.606 | 28.5 | 0.081 | 3.8 |
| 0.3 | 0.780 | 35 | 0.113 | 5.1 | 0.631 | 30 | 0.087 | 4.1 |
| Control | 0.739 | 33 | 0.098 | 4.4 | 0.594 | 28 | 0.089 | 4.2 |
| 100% | 2.23 | | | | 2.12 | | | |

| C'1 esterase PNH cells Esterase units added | pH 6.5 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Case 4 | | Case 5 | | Case 7 | | Case 8 | | Case PE | |
| | OD 540 | % lysis | OD 540 | % lysis | OD 540 | % lysis | OD 540 | % lysis | OD 540 | % lysis |
| 1 | 1.112 | 48.8 | 0.940 | 68.5 | 0.639 | 33.2 | 0.370 | 19.4 | 0.538 | 32.4 |
| 2 | 1.623 | 71.3 | 1.023 | 74.5 | 1.023 | 53.2 | 0.530 | 27.8 | 0.629 | 38.5 |
| 3 | 1.960 | 86 | 1.241 | 90.5 | 1.178 | 61.2 | 0.588 | 30.8 | 0.700 | 42 |
| 4 | 2.02 | 88.6 | 1.277 | 93 | 1.305 | 67.8 | 0.629 | 32.9 | 0.724 | 43.5 |
| 6 | 2.14 | 94 | 1.324 | 96.5 | 1.234 | 64.2 | 0.613 | 32.1 | 0.715 | 43 |
| 10 | 1.512 | 66.4 | 1.230 | 89.5 | 0.708 | 36.8 | 0.621 | 32.5 | 0.700 | 42 |
| 15 | 0.970 | 42.5 | | | 0.461 | 23.9 | 0.501 | 26.2 | 0.625 | 38 |
| 30 | | | 0.409 | 29.7 | | | | | | |
| Control | 0.925 | 40.6 | 0.805 | 58.5 | 0.474 | 24.6 | 0.280 | 14.7 | 0.470 | 28 |
| 100% | 2.28 | | 1.376 | | 1.925 | | 1.910 | | 1.658 | |

| Streptokinase PNH cells Units added | pH 6.5 | | Aggregated γ-globulin PNH cells μg added | pH 6.5 | |
|---|---|---|---|---|---|
| | OD 540 | % lysis | | OD 540 | % lysis |
| 1,000 | 0.865 | 46 | 4,300 | 0.002 | 0 |
| 500 | 1.008 | 54 | 43 | 0.451 | 29 |
| 100 | 1.196 | 64 | 21.5 | 0.875 | 46.5 |
| 50 | 1.341 | 72 | 14.3 | 0.960 | 51 |
| | | | 10.8 | 1.051 | 56 |
| Control | 0.800 | 42 | 8.6 | 0.985 | 53 |
| 100% | 1.870 | | | | |
| | | | Control | 1.838 | 44 |
| | | | 100% | 1.870 | |

TABLE II—(*Continued*)

| Dextran sulfate μg added | pH 6.5 | | | | | |
|---|---|---|---|---|---|---|
| | Dextran sulfate 60 S | | Dextran sulfate 200 S | | | |
| | PNH cells | | PNH cells | | Normal cells | |
| | OD 540 | % lysis | OD 540 | % lysis | OD 540 | % lysis |
| 500 | 0.044 | 2.2 | .880 | 43 | 0 | 0 |
| 250 | 1.460 | 73.8 | 1.575 | 77 | 0.207 | 9.7 |
| 125 | 1.490 | 75.4 | 1.660 | 81 | 0.363 | 17.0 |
| 62.5 | 1.280 | 64.6 | 1.310 | 64 | 0.055 | 2.6 |
| 31 | 0.980 | 49.4 | 0.965 | 47 | 0.006 | 0 |
| 16 | 0.850 | 42.8 | 0.770 | 37.6 | 0.006 | 0 |
| 8 | 0.805 | 40.6 | 0.750 | 36.6 | 0.010 | 0.05 |
| Control | 0.800 | 40.3 | 0.740 | 36 | 0.002 | 0 |
| 100% | 1.980 | | 2.05 | 91 | 2.13 | |

Simultaneous test of all reagents—pH 6.5

| Reagent | Amount | PNH cells | | Normal cells | |
|---|---|---|---|---|---|
| | | OD 540 | % lysis | OD 540 | % lysis |
| Poly I | 0.7 μmole | 1.750 | 90.6 | 1.123 | 53 |
| Dextran sulfate | 125 μg | 1.480 | 76.6 | 0.354 | 16.7 |
| C'1 esterase | 6 U | 0.900 | 46.6 | 0.010 | 0.4 |
| Streptokinase | 50 U | 0.772 | 40 | 0.000 | 0 |
| Aggregated γ-globulin | 10.8 μg | 0.754 | 39 | 0.010 | 0.4 |
| Control | | 0.680 | 35.2 | 0.000 | 0 |
| 100% | | 1.930 | | 2.120 | |

C'1a, C'1 esterase, streptokinase, and aggregated γ-globulin (Figure 2B, Table II).

*Effect of pre-exposure of red cells to test material upon their lytic susceptibility.* Certain agents capable of altering the red cell membrane are able to render NHE susceptible to C'-dependent hemolysis in acidified human serum (16). In addition, red cells coated by antigen are susceptible to C'-dependent hemolysis in the presence of specific antibody (20). Experiments were performed to investigate the possibility that poly I-induced hemolysis might share a similar mechanism, i.e., that poly I was altering, sensitizing, or attaching to the red cell surface in a manner analogous to the action of influenza virus, proteolytic enzymes, neuraminidase, periodate ions, tannic acid (21), or "antigen." One-tenth ml of a 20% suspension of PNHE or NHE was exposed to 0.5 ml poly I solution (10 μmoles and 4 μmoles P per ml) at 37° C for 30 minutes. After washing 3 times in 0.15 M NaCl, they were subjected to hemolysis in human serum at pH 6.5 and compared with control cells exposed to saline solution only. There is no change in the susceptibility of PNHE to hemolysis after pre-exposure to poly I, nor do NHE develop any hemolytic properties after such treatment. Similar results were obtained using dextran sulfate (1,250 μg per ml), C'1 esterase (60 U per ml), aggregated γ-globulin (108 μg per ml), and streptokinase (500 U per ml). Thus the mechanism by which these materials provoke or enhance hemolysis does not depend on their ability to attach to, alter, or sensitize the red cell membrane; this implies that an explanation for their mechanism of action should be sought in relation to their effect upon serum factors involved in red cell lysis.

*Effect of pre-exposure of serum to test material upon the capacity of test material to induce or enhance hemolysis.* During the initial phase of these studies poly I occasionally failed to stimulate PNHE hemolysis. It was finally appreciated that this failure occurred whenever poly I and serum had been in contact for an extended period before addition of cells. Only after the need for simultaneous addition of poly I and red cells had been appreciated was the ability of poly I to provoke substantial hemolysis of NHE at pH 6.5 first recognized. The experiments defining the kinetics of both hemolytic systems with the passage of time between poly I and red cell addition to serum are shown in Figure 3. At pH 6.5 the ability of poly

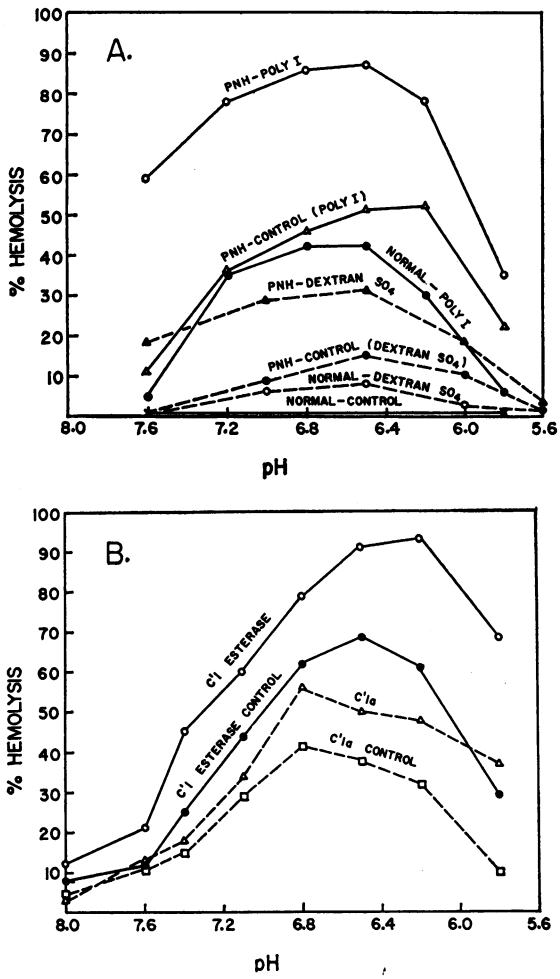


FIG. 2A. EFFECT OF pH UPON POLY I AND DEXTRAN SULFATE-INDUCED LYSIS. 1.0 μmole poly I or 125 μg dextran sulfate was employed. B. EFFECT OF pH UPON C'1A AND C'1 ESTERASE ENHANCEMENT OF PNH RED CELL HEMOLYSIS. Six U C'1 esterase (0.1 ml) and 9.6 U C'1a (0.4 ml) were employed. PNH = paroxysmal nocturnal hemoglobinuria.

I to enhance PNHE hemolysis is lost after 10 minutes preincubation, and thereafter the serum-poly I mixture gradually loses all ability to sustain PNHE hemolysis, total inhibition occurring at 45 minutes. At pH 7.6, although the degree of hemolysis is initially less than at pH 6.5, the rate of decline of poly I enhancement is much slower. The hemolytic system involving NHE is most sensitive to the duration of poly I-serum preincubation; by 8 minutes practically all hemolytic capacity has been lost. Similar results were obtained with the other test materials (Table III).

*Effect of serum dilution upon poly I-induced hemolysis.* The PNH hemolytic system is known to be highly sensitive to serum dilution (22). Figure 4 demonstrates that poly I enhancement of PNHE hemolysis is also destroyed by a 1:4 dilution of serum both at pH 6.5 and 7.6. The induction of NHE hemolysis by poly I is even more sensitive to serum dilution, being abolished almost totally by a 1:1.6 dilution of serum. Similar results, in the case of PNHE hemolysis, were obtained using C'1 esterase (Table IV).

*Effect of red cell storage upon susceptibility to poly I-induced hemolysis.* Certain red cell agglutinins are known to attack preferentially cells that have been stored for prolonged periods of time *in vitro* (23). NHE kept aseptically in ACD solution for 4 weeks at 4° C were repeatedly tested for their susceptibility to poly I-induced hemolysis using a single serum pool. Little, if any, increase in hemolytic susceptibility is noted with time (Table V).

*Effect of EDTA salts upon poly I-induced hemolysis.* PNHE hemolysis is known to be dependent on $Mg^{++}$, but $Ca^{++}$ ion is not required (8). $Na_3HEDTA$ (which binds both $Ca^{++}$ and $Mg^{++}$) and $Na_2MgEDTA$ (which binds $Ca^{++}$, but not $Mg^{++}$) were used to investigate the divalent cation requirements for poly I-induced hemolysis. $Na_3HEDTA$ completely abolished poly I-induced hemolysis, whereas $Na_2MgEDTA$ diminished but did not destroy the capacity of poly I to enhance hemolysis of both PNHE and NHE (Table VI). These results indicate that these hemolytic systems display an absolute requirement for $Mg^{++}$, but not for $Ca^{++}$.

*Effect of serum complement component or serum properdin depletion upon poly I-induced hemolysis.* Since PNHE hemolysis is known to be dependent on all four components of C′ and properdin (8), and since poly I is known to profoundly affect the C′ system (1, 4, 5), the ability of heat inactivated serum and of various R reagents to support poly I-induced hemolysis was investigated. Serum heated at 56° C for 30 minutes no longer supports red cell lysis even when poly I is added. In addition, treatment of serum so as to remove any one of the four major C′ components renders such an R reagent devoid of the ability to support hemolysis of either PNHE

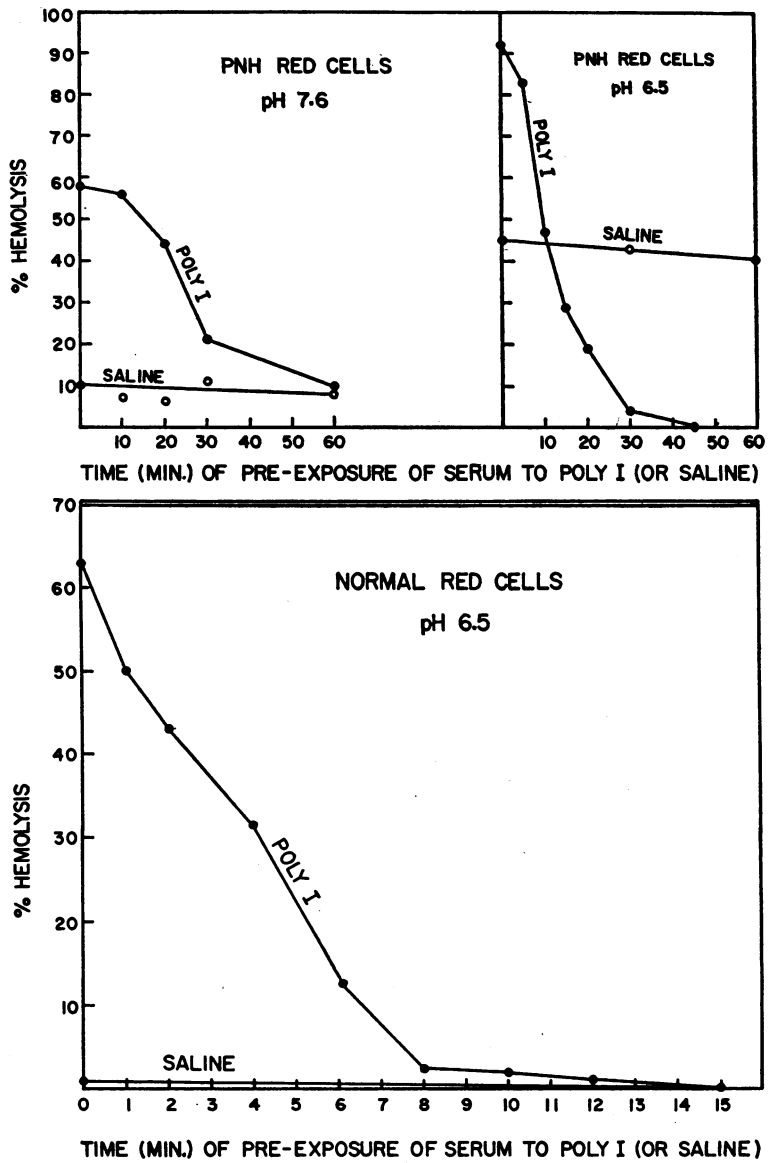


FIG. 3. EFFECT OF PRE-EXPOSURE OF SERUM TO POLY I UPON THE CAPACITY OF POLY I TO ENHANCE PNH RED CELL LYSIS (UPPER CURVES) OR INDUCE NORMAL RED CELL LYSIS (LOWER CURVE). One-tenth ml saline or poly I (1.0 μmole P) was added to 1 ml serum at the designated pH. After the indicated time interval 0.05 ml of a 20% suspension of red cells was added and the amount of hemolysis occurring at 37° C for 30 minutes determined.

or NHE, and the addition of poly I does not repair this deficiency (Table VII). The experiments involving NHE hemolysis in R4 were handicapped by the low level of control hemolysis observed with poly I addition, since this control was carried out at a serum dilution of 1 : 1.45 to correspond with the dilution of R4. From these experiments it seems likely that the ability of poly I to enhance or initiate red cell hemolysis is mediated through the C' system, and in all likelihood is an earlier stage of the same process which, with the passage of time, leads to inactivation of the PNHE hemolytic system. Removal of properdin inhibits the capacity of serum to support PNHE

TABLE III

*Effect of pre-exposure of serum to various test materials upon their capacity to induce or enhance human red cell lysis*

| Test material and amount used | Red cells employed* | pH of serum | Time of pre-incubation of serum and test material | OD 540 | % lysis |
|---|---|---|---|---|---|
| | | | *min* | | |
| A. Dextran sulfate | PNHE | 6.5 | 0 | 1.435 | 74.3 |
| 125 μg | | | 1 | 1.400 | 72.5 |
| | | | 3 | 0.660 | 34.2 |
| | | | 5 | 0.103 | 5.3 |
| | | | 15 | 0.070 | 3.6 |
| | | | 30 | 0.065 | 3.4 |
| Saline control | | | 0 | 0.690 | 35.8 |
| Saline control | | | 30 | 0.570 | 29.5 |
| 100% | | | | 1.930 | |
| B. Dextran sulfate | NHE | 6.5 | 0 | 0.353 | 16.7 |
| 125 μg | | | 1 | 0.285 | 13.4 |
| | | | 3 | 0.002 | 0 |
| | | | 5 | 0.002 | 0 |
| Saline control | | | 0 | 0 | 0 |
| Saline control | | | 5 | 0 | 0 |
| C. C'1a | PNHE | 6.5 | 0 | 0.930 | 55 |
| 9.6 U (added | | | 0.5 | 0.743 | 44 |
| in 0.4 ml | | | 1 | 0.479 | 28 |
| saline) | | | 3 | 0.205 | 12 |
| | | | 5 | 0.169 | 10 |
| | | | 10 | 0.157 | 9.3 |
| | | | 30 | 0.112 | 6.6 |
| | | | 60 | 0.078 | 4.6 |
| Saline control | | | 0 | 0.437 | 26 |
| Saline control | | | 30 | 0.361 | 21.3 |
| Saline control | | | 60 | 0.348 | 20.5 |
| 100% | | | | 1.695 | |
| D. C'1 esterase | PNHE | 6.5 | 0 | 1.270 | 97 |
| 6 U | | | 0.5 | 1.144 | 87.5 |
| | | | 1 | 0.839 | 64 |
| | | | 3 | 0.438 | 33 |
| | | | 5 | 0.421 | 32 |
| | | | 10 | 0.379 | 29 |
| | | | 15 | 0.316 | 24 |
| | | | 20 | 0.252 | 19 |
| | | | 30 | 0.233 | 17.5 |
| D. Saline control | | | 0 | 0.816 | 62 |
| Saline control | | | 30 | 0.576 | 44 |
| 100% | | | | 1.313 | |
| E. Streptokinase | PNHE | 6.5 | 0 | 1.011 | 69 |
| 1,000 U | | | 1 | 0.990 | 67 |
| | | | 3 | 0.457 | 31 |
| | | | 5 | 0.246 | 16.5 |
| | | | 10 | 0.132 | 8.9 |
| | | | 20 | 0.063 | 4.2 |
| | | | 30 | 0.035 | 2.4 |
| | | | 60 | 0.043 | 2.9 |
| Saline control | | | 0 | 0.813 | 55 |
| Saline control | | | 60 | 0.802 | 54 |
| 100% | | | | 1.482 | |
| 50 U | PNHE | 6.5 | 0 | 1.255 | 76 |
| | | | 5 | 1.198 | 73 |
| | | | 10 | 1.000 | 61 |
| | | | 15 | 0.795 | 48 |
| | | | 30 | 0.695 | 42 |
| Saline control | | | 0 | 0.950 | 58 |
| Saline control | | | 30 | 0.945 | 57.5 |
| 100% | | | | 1.640 | |
| F. Aggregated | PNHE | 6.5 | 0 | 1.243 | 76 |
| γ-globulin | | | 0.5 | 1.183 | 72 |
| | | | 1 | 1.032 | 63 |
| 10.8 μg | | | 3 | 0.995 | 60 |
| | | | 5 | 0.805 | 49 |
| | | | 10 | 0.720 | 44 |
| | | | 15 | 0.631 | 38 |
| | | | 20 | 0.593 | 36 |
| | | | 30 | 0.508 | 31 |
| Saline control | | | 0 | 0.950 | 58 |
| Saline control | | | 30 | 0.945 | 57.5 |
| 100% | | | | 1.640 | |

* PNHE = red cells from patients with paroxysmal nocturnal hemoglobinuria; NHE = normal human red cells.

hemolysis. The ability to hemolyze PNHE can be restored to RP serum by the addition of poly I; removal of properdin from serum diminishes, but does not completely abolish, the ability of poly I to provoke hemolysis of NHE (Table VIII).

TABLE IV

*Effect of serum dilution upon the enhancement of PNHE hemolysis by C'1 esterase*

| Serum dilution | Saline control | | C'1 esterase | |
|---|---|---|---|---|
| | OD 540 | % lysis | OD 540 | % lysis |
| Undiluted | 0.920 | 49.1 | 1.532 | 82 |
| 1:1.2 | 0.922 | 49.2 | 1.417 | 75.7 |
| 1:1.4 | 0.960 | 51.3 | 1.312 | 70.2 |
| 1:1.6 | 0.950 | 50.8 | 1.037 | 55.5 |
| 1:1.8 | 0.880 | 47 | 0.830 | 44.3 |
| 1:2 | 0.798 | 42.7 | 0.655 | 35 |
| 1:3 | 0.275 | 14.7 | 0.138 | 7.4 |
| 100% | 1.870 | | | |

*Effect of antigen-antibody precipitate upon the PNH hemolytic system.* The effect of an antigen-

TABLE V

*Effect of red cell storage upon susceptibility to poly I-induced lysis*

| Date of test | % lysis (pH 6.5) | |
|---|---|---|
| | Control | Poly I |
| 3/23 | 0 | 54 |
| 3/26 | 0.6 | 63 |
| 3/30 | 1.2 | 52 |
| 4/6 | 1.1 | 61 |
| 4/13 | 2.0 | 65 |
| 4/21 | 0 | 64.5 |

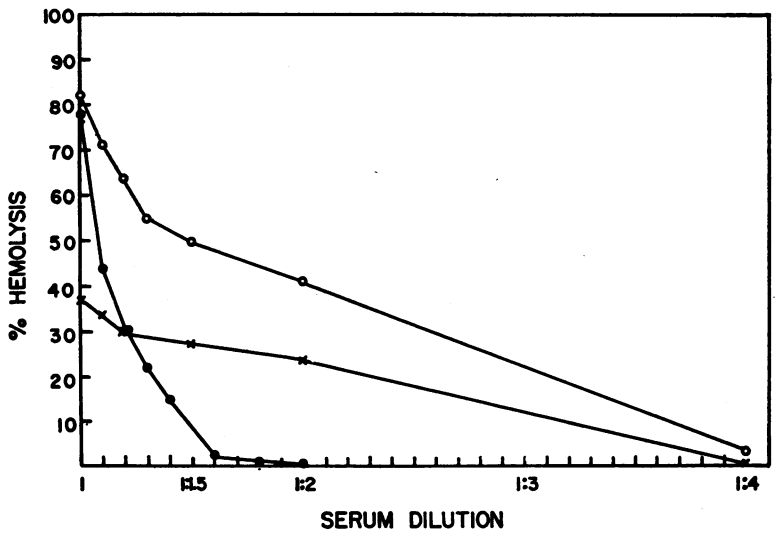


FIG. 4. EFFECT OF SERUM DILUTION UPON POLY I-INDUCED HEMOLYSIS. Serum (pH 6.5) was diluted as indicated with 0.15 M NaCl. One μmole P poly I was employed as the stimulating test dose. The saline control for normal red cells showed no hemolysis and is therefore not plotted. PNH red cells–saline control, ×——×; PNH red cells–poly I, ○——○; normal red cells–poly I, ●——●.

antibody precipitate on the PNHE hemolytic system was examined. No enhancement of PNHE hemolysis was observed over a wide dose range. Large amounts are capable of destroying serum factors involved in PNHE hemolysis progressively with the passage of time.

*Effect of C'1 esterase serum inhibitor upon poly I- and C'1-induced hemolysis.* Two and three-tenths U C'1 EI preadded to serum was incapable of preventing the enhancement or induction of hemolysis by poly I. Preincubation of C'1a or C'1 esterase with stoichiometric amounts of C'1 EI abolished their ability to enhance PNHE hemolysis.

## Discussion

During classical immune lysis all the steps intermediate between coating of the cell with anti-

TABLE VI

*Effects of various salts of EDTA upon poly I-induced hemolysis**

| Test description | Test constituents | | | | | | Results | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | PNHE | | NHE | |
| | 20% red cells | Serum pH 6.5 | Poly I 10 μmoles P/ml | $Na_2HEDTA$ 0.15 M | $Na_2MgEDTA$ 0.15 M | Saline | OD 540 | % lysis | OD 540 | % lysis |
| | *ml* | *ml* | *ml* | *ml* | *ml* | *ml* | mμ | | mμ | |
| Saline control | 0.05 | 1.0 | | | | 0.25 | 0.564 | 46 | 0.022 | 1.3 |
| Poly I | 0.05 | 1.0 | 0.1 | | | 0.15 | 1.093 | 91 | 0.614 | 37 |
| $Na_2HEDTA$ | 0.05 | 1.0 | | 0.15 | | 0.1 | 0.074 | 6.1 | 0.0 | 0 |
| $Na_2HEDTA$ poly I | 0.05 | 1.0 | 0.1 | 0.15 | | | 0.066 | 5.5 | 0.0 | 0 |
| $Na_2MgEDTA$ | 0.05 | 1.0 | | | 0.15 | 0.1 | 0.562 | 46 | 0.005 | 0.3 |
| $Na_2MgEDTA$ poly I | 0.05 | 1.0 | 0.1 | | 0.15 | | 0.960 | 80 | 0.350 | 21 |
| Blank | | 1.0 | | | | 0.3 | | | | |
| 100% (Freeze and thaw) | 0.05 | 1.0 | | | | 0.25 | 1.197 | | 1.665 | |

* The final concentration of EDTA in the test mixtures is $1.7 \times 10^{-2}$ M.

TABLE VII

*Effect of complement component depletion upon poly I-induced hemolysis**

| | Reagent tested | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | R1 | R1† + H.S. | R2 | R2† + H.S. | R3 | R4 | Normal serum | Normal‡ serum | Normal§ serum |
| | | | | | % hemolysis | | | | |
| PNHE saline | 3.7 | 1.9 | 1.2 | 5 | 1.2 | 5.5 | 43 | 42.5 | 40 |
| PNHE poly I | 3.4 | 9.0 | 0 | 4 | 2.6 | 6.5 | 97.3 | 88 | 67 |
| NHE saline | 4 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| NHE poly I | 1 | 4 | 0 | 1 | 3 | 1 | 54 | 26 | 9 |

* Normal serum and R reagents from same AB serum pool. All tests performed at pH 6.5.
† Two parts R reagent + 1 part heated serum (H.S.), 56° C, 30 minutes.
‡ Normal serum diluted 1:1.25 with 0.15 M NaCl; for comparison with R1.
§ Normal serum diluted 1:1.45 with 0.15 M NaCl; for comparison with R4.

body and actual E* formation occur at the cell surface (24). Thus the presence of oriented antibody molecules arranged in proper spatial conformation serves as a site of attachment for (11), and, through an allosteric mechanism, as an activator of, the C'1 molecule. The latter in turn proceeds to direct the attachment and *in situ* activation of C'4 and C'2, and so forth. The fact that the entire process occurs sequentially in direct apposition to the ultimate target for C' action, the cell membrane, insures the greatest efficiency; in a sense the sensitized red cell surrounds itself with a halo of C' activity. Although the availability of fluid phase C' components for participation in this spatially localized sequence of events depends upon random phenomena, the sequential process of activation insures that all of the SA at which the entire sequence of C' component attachment and activation occurs will result in S* formation.

Certain of the reactions that proceed at the cell membrane during the formation of EAC'. . . intermediates have their counterpart in events that can proceed entirely in the fluid phase. Thus it has long been recognized that purified activated C'1 or C'1 esterase attack fluid phase C'4 and C'2 (12, 25). Such an interaction has usually been detected by the fact that C'4 and C'2 are thus rendered hemolytically inert. However, recent evidence has indicated that C'1 esterase can, under appropriate conditions, enhance the activity of C'2 in the fluid phase (25) presumably by a process analogous to the $C'2 \xrightarrow{C'1a} C'2a$ conversion that occurs at the surface of an $EAC'_{1,4}$ complex (26).

The unique capacity of poly I alone of all the polynucleotides tested to enhance hemolysis of PNHE or to initiate hemolysis of NHE is reminiscent of the ability of this material, also unique, to serve as an anti-C' substance (1). In addition, the same maneuver that renders poly I incapable of interacting with whole C' (1), C'1, or C'1q (4), and which destroys its capacity to evoke vascular permeability (6), helix formation with poly A or poly C, also destroys its capacity to enhance or induce hemolysis. Pre-exposure of red cells to any of the agents employed does not increase their susceptibility to hemolysis upon subsequent incubation with human serum; this suggests that the ability of these agents to stimulate hemolysis depends upon their interaction with serum components and not upon any capacity to attach to, sensitize, or alter the red cell. The failure of heat-inactivated serum, or serum deficient in any one of the four major subcomponents of C', to support hemolysis in the presence of polyinosinic acid suggests that the serum components involved in poly I-induced hemolysis are those of the C' system. Certainly the abolition of poly I-induced hemolysis by chelation of $Ca^{++}$ and $Mg^{++}$ ($Na_3$-

TABLE VIII

*Effect of properdin depletion upon the ability of poly I to induce or enhance human red cell lysis**

| | % hemolysis | |
|---|---|---|
| | NHE | PNHE |
| RP serum + saline | Not done | 2 |
| RP serum + poly I (1 μmole P) | 18 | 64 |
| Normal serum + poly I (1 μmole P) | 49.5 | 81 |
| Normal serum + saline (control) | 0 | 51 |

* The tests were performed at pH 6.5.

HEDTA) is consistent with C' mediation, since the dependence of C' activity on the presence of these cations has been amply demonstrated (27). The ability of poly I-induced hemolysis to proceed in the presence of $Na_2MgEDTA$ is disturbing since this chelating salt, which binds $Ca^{++}$ but not $Mg^{++}$, has been shown to destroy the capacity of poly I to interact with the whole C' system (5). However, the latter observations were made in a dilute C' reagent. Subsequent studies in this laboratory have shown that the need for $Ca^{++}$ in immune lysis is not absolute; EA will undergo maximal lysis in undiluted human serum or in serum diluted 1:2 even in the presence of $Na_2$-MgEDTA (28). Apparently sufficient C'1a can be generated under such circumstances to initiate and sustain the sequence of events leading to C'-dependent hemolysis. Presumably, in undiluted human serum as a C' source, the dependence of the poly I-C'1 interaction upon $Ca^{++}$ is also not absolute.

Thus materials that under certain circumstances (immune lysis, dilute C') act to inhibit C'-dependent lysis, under other circumstances (non-immune lysis, undiluted C') act to enhance or initiate C'-dependent hemolysis. The mechanism of inhibition of immune lysis by poly I, aggregated gamma globulin (29, 30), and streptokinase (31, 32) has been well established; dextran sulfate has been less carefully studied, but probably functions in a similar manner (33, 34). To explain the second phenomenon we have formulated the following hypothesis: the addition of these materials to serum initiates the evolution of fluid phase C'1a. Although the C'1a thus formed is incapable of attaching to NHE (and probably to PNHE), both by virtue of the lack of a proper site of attachment on the cell (antibody), and through binding of the C'1q (11 S) portion of the molecule (in the cases of aggregated $\gamma$-globulin and poly I), it can initiate a sequence of events involving the remainder of the fluid phase components of the C' system that results in red cell damage and hemolysis. This phase of the process, which might be termed the "activation" phase, can be extended to involve C'4 and C'2 on the basis of our current understanding of the mechanisms involved. A similar process of activation of fluid phase C'3 subcomponents [$\beta_{1c}$-globulin (35); C'3c, C'3b (36)] by C'2a in the presence of $Mg^{++}$ could then be postulated, with the ultimate appearance in the fluid phase of C'-dependent hemolytic factors. This process, with the passage of time, would be expected to dissipate itself, since at least one of the fluid phase reactions involved ($C'2 \xrightarrow{C'1a} C'2a$), if allowed to proceed, finally results in the formation of a hemolytically inert form of C'2 (37). The recent observations of Müller-Eberhard, Calcott, and Mardiney on the ability of cell-bound C'2a to inactivate $\beta_{1c}$-globulin are also pertinent to this argument (38). This second phase, the "inactivation" phase, would explain the fact that with longer periods of preincubation of poly I and serum, the ability to initiate NHE lysis is lost and PNHE lysis is ultimately inhibited. Similar fluid phase events can both stimulate and inhibit classical immune hemolysis. Haines and Lepow have shown that properly chosen amounts of C'1 esterase can enhance the conversion of $EAC'_{1,4}$ to $EAC'_{1,4,2a}$ by R3. The efficiency of this enhancement diminishes as the period of R3 pre-exposure to C'1 esterase is prolonged. With larger amounts of C'1 esterase, enhancement is entirely abolished and only inhibition is noted (25). We have chosen to describe these phenomena as the production of red cell membrane damage via "indifferent" activation of the C' system, since no antigen-antibody reaction specific for the red cell is involved. These hemolytic systems are clearly different from those reported by Neter and his co-workers (20, 39, 40), by Boyden and Andersen (41), and by Middlebrook (42), since in these latter systems antigen (or antigen-antibody complex) is capable of adsorbing to the red cell surface, and thus acts as a red cell sensitizer. In addition, these authors have all noted that normal serum in high concentration interferes with red cell adsorption of the sensitizing material (39–43). The hemolytic system described by Cowan (44) involving polyethylene glycol, guinea pig C', and sheep red cells is in many respects directly comparable to the present systems. Although red cells pre-exposed to polyethylene glycol and subsequently washed are not susceptible to hemolysis, the author postulates that polyethylene glycol acts as a red cell sensitizer since it forms a precipitate when mixed with an emulsion of Forssman hapten prepared from red

cell stroma. At the same time the author notes the ability of polyethylene glycol to interact with C'1 when added to undiluted guinea pig serum; thus polyethylene glycol-induced hemolysis may well be effected through the mechanism outlined above. An analogous system involving *in vitro* damage to platelets by the C' system initiated by an unrelated antigen-antibody reaction has recently been described (45). We have as yet failed to initiate a similar sequence of events vis-à-vis red cell damage by antigen-antibody precipitate. On the other hand, substantial confirmation of the theory presented has been achieved by use of partially purified C'1a and C'1 esterase, since addition of these materials to human serum will substantially enhance PNH hemolysis in the early phase of C'1a-serum contact, only to be followed by an "inactivation" phase similar in all respects to the phenomenon induced by poly I and the other test materials. Since C'1 esterase lacks a binding site and cannot induce $EAC'_1$ formation (3, 25), the mechanism of its action must certainly be sought in relationship to fluid phase events.

The extreme sensitivity of test material-induced hemolysis to dilution may result from the fact that these systems, like the native PNHE hemolytic system, lack a mechanism for selectively and efficiently surrounding the cell, over an extended period, with those activated C' components directly involved in membrane damage; instead, both systems depend upon random "hits" inflicted on the membrane by transiently activated fluid phase C' components. Jenkins, Christenson, and Engle have demonstrated that those PNHE resistant to hemolysis in the native acid hemolysis test have become coated with components of the C' system (46). They have found that such cells react readily with anti-$\beta_{1c}$-globulin (anti-C'3) but not with anti-$\beta_{1e}$ (47)-globulin (48) (anti-C'4). In this connection it is of interest that Rosen has recently shown, using $I^{131}$-labeled $\beta_{1c}$-globulin, that the PNHE, in contrast to the NHE, has the capacity to coat itself with $\beta_{1c}$-globulin without the mediation of other fluid phase C' components (49). Such results imply that when the mechanism of red cell lysis by C' depends largely on fluid phase components, the late reacting C' component may attach directly to the cell membrane.

If PNHE hemolysis is primarily dependent upon fluid phase, late-acting, C' components, can one view PNHE as if they were red cell-C' component intermediates? The fact that PNHE hemolyze in the presence of $Na_2MgEDTA$ might suggest that they are in the state $PNH\text{-}EC'_1$ or $PNH\text{-}EC'_{1,4}$. However, as mentioned earlier, EA will also hemolyze under similar conditions (28). In addition, after pre-exposure to $Na_3$-HEDTA, a procedure that would elute any cell bound C'1 (50), PNHE show unaltered capacity to hemolyze in both native and $Na_2MgEDTA$ serum (28). PNHE will not hemolyze in R4 (8) nor in serum containing $Na_3HEDTA$ (28), eliminating the possibility that they are in the state $PNH\text{-}EC'_4$ or $PNH\text{-}EC'_{1,4,2}$. Thus, PNHE not only lack antibody, but in their native state also lack C' component coats. The latter only appear after *in vitro* acid hemolysis (46), or possibly during severe hemolytic episodes *in vivo,* since the incidence of positive Coombs test appearing sometime during the course of the disease is higher than would be expected simply on the basis of random occurrence (51, 52).

Test material-induced hemolysis is more pronounced when carried out at pH 6.5 than at pH 7.6, and resembles in this respect the native PNHE hemolytic system. In addition, other pH-dependent hemolytic systems such as are found in high titer cold agglutinin disease (53) and the *in vitro* hemolytic system involving artificially altered red cells (16) have been described. Recent studies in this laboratory have shown that the optimal pH for immune hemolysis is also 6.5 when human serum is the source of C' (54). Thus the fact that these several C'-dependent hemolytic systems function best at pH 6.5 can no longer be used to distinguish between them and classical EA hemolysis. The relative $C'H_{50}$ titer of a human C' source when measured at pH 6.5 is approximately 1.6 times that found at pH 7.5; studies with EAC'. . . have shown that most of this increase can be attributed to the late phase reaction $EAC'_{1,4,2} + C'3 \longrightarrow E^*$ (54). The striking effect of pH change on the poly I-induced hemolysis of NHE can be better understood if one reasons that changing the pH from 6.5 to 7.6 is, in effect, like diluting the serum at pH 6.5 1 : 1.6. In both instances hemolytic capacity is almost entirely lost. The stimulatory effect of pH alteration upon native PNHE hemolysis is different in

mechanism from the effects of C'1 activators or C'1 esterase. Titrating serum from pH 7.6 ⟶ 6.5 enhances its hemolytic potential, but serum kept at pH 6.5 for extended periods does not display a pronounced "inactivation" phase. In addition, restoring the pH to 7.6 entirely abolishes the enhanced hemolytic potential both for PNHE cells and EA (54). We have concluded that PNHE hemolysis is, in a sense, a threshold phenomenon. The PNHE, injured in some unknown way, is extremely susceptible to C'-dependent injury. Normally, a small amount of fluid phase C'-derived hemolytic potential may be present in serum by virtue of an intrinsic low-grade "activation-inactivation" mechanism. Increasing the potency of the late-acting components of this intrinsic fluid phase system by lowering the pH suffices to lyse PNHE. NHE require either more intense fluid phase activation (poly I, dextran sulfate) or a membrane-localized C' activation process (antibody), or both. PNHE also display their heightened sensitivity to C'-dependent hemolysis in the fluid phase-activated hemolytic systems and in C'-dependent lysis provoked in the usual way by a variety of red cell antibodies (55, 56).

Recognizing that red blood cells can be destroyed by the C' system through the process of "indifferent" C' activation implies that such a mechanism may initiate or exacerbate certain hemolytic anemias in man. Thus the well-known propensity of patients with acquired hemolytic anemia, including PNH, to be worsened by bouts of intercurrent infection might be related to immune interactions between antibody and the invading agent or its products that indifferently activate the C' system. Indeed, such a mechanism might be sufficient to explain the pathogenesis of certain acquired hemolytic anemias in man. Lupus erythematosus, for example, is a disease in which acquired hemolytic anemia is a common finding (57). The similarity between the renal lesions in lupus patients and those produced by experimental hyperimmunization of rabbits is striking (58a). Christian, Hatfield, and Chase have recently shown that circulating antigen-antibody complexes may be present in the serum of lupus erythematosus patients (59); such complexes, acting in a fashion similar to poly I, might result in C' coating of, or injury to, red cells. Any Coombs-positive acquired hemolytic anemia wherein the red cell coating substance(s) is restricted to non-gamma (presumably C') factors, where red cell antibodies are not demonstrable, is potentially caused by indifferent C' activation (58b, 58c, 60). Paroxysmal nocturnal hemoglobinuria may be due entirely to such a mechanism. C' activation, perhaps by such complexes as have been recently described in a significant number of PNH patients (61), might be entirely responsible for all the manifestations of red cell injury, both *in vivo* and *in vitro,* seen in this disease. The demonstration that cells can suffer damage and destruction by the C' system without the mediation of specific anticell antibody suggests that autoimmune mechanisms need not necessarily be invoked to explain the presence of C' immunoglobulins on cells (58a, 58b) or in tissue (62, 63).

## Summary

The addition of polyinosinic acid (poly I) to a suspension of red cells from patients with paroxysmal nocturnal hemoglobinuria (PNH) in undiluted normal human serum results in pronounced stimulation of red cell lysis. In addition poly I, under similar circumstances, will initiate substantial hemolysis of normal human red cells. Poly I loses the ability to induce hemolysis if it is combined with polyadenylic or polycytidylic acids to form a hydrogen bonded helical structure. The pH optimum for poly I-induced hemolysis is 6.5. Exposure of red cells to poly I, followed by thorough washing, does not result in increased red cell hemolysis when the cells are subsequently added to serum. Exposure of serum to poly I for increasing periods of time results in progressive decrease in the amount of hemolysis produced upon subsequent addition of red cells. Serum pre-exposed to poly I ultimately loses the ability to support any hemolysis of PNH red cells. Poly I-induced hemolysis is rapidly lost upon slight dilution of serum, but is not affected by prolonged *in vitro* storage of red cells. Poly I-induced hemolysis is abolished by addition to serum of $Na_3$-HEDTA, but is not affected by $Na_2MgEDTA$. Removal from serum of any one of the four major components of complement abolishes its capacity to support poly I-induced hemolysis. Removal of properdin from serum only partially reduces its capacity to support poly I-induced hemolysis.

Other activators of the first component of complement (C'1) such as dextran sulfate, streptokinase, and aggregated gamma globulin, as well as activated C'1 and C'1 esterase, can enhance PNH red cell lysis in a similar fashion. Dextran sulfate can also induce normal red cell lysis. These hemolytic systems are presumed to result from the evolution in the fluid phase of hemolytically active, late-acting, labile complement components, capable of injuring red cell membrane despite the lack of an antibody coat. The observation that "indifferent" complement activation can result in red cell damage may be pertinent to concepts regarding the pathogenesis of certain acquired hemolytic anemias in man.

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