Inhibitors of Complement Derived From the Erythrocyte Membrane in Paroxysmal Nocturnal Hemoglobinuria

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Extracts of the membranes of normal red cells and red cells from all subpopulations of paroxysmal nocturnal (PNH) red cells inhibited antibody-mediated complement activation. These extracts were shown to accelerate decay of the complement complex, C42, and the relative amount of inhibitory activity was similar in normal and PNH membranes. Inhibitors derived from normal red cells markedly decreased lysis of both PNH and normal cells when antibody was present in excess and complement was limiting. These same inhibitors decreased PNH cell lysis to a much lesser degree when complement was activated with cobra venom or acidified serum. The susceptibility of the PNH cell to complement lysis because of an increased fixation of C3 to its membrane is not due to a difference in membrane-associated accelerator of the decay of the C42 complex.

The abnormal red cells of patients with paroxysmal nocturnal hemoglobinuria (PNH) are characteristically more easily lysed by complement than are normal cells. This is due in part to an increase in fixation of C3 to the cell membrane. C3 may be fixed by either of two complexes: C42 (activated by specific antibody and C1) and C3bBb (activated through the alternative pathway, as in acidified normal serum, or in serum treated with a protein factor in cobra venom. Extracts from human red cell stromata have been found to cause inhibition of complement-mediated lysis of antibody-sensitized sheep red cells. This inhibitor, I-H, was found to interfere not only with C42 activity but also with activation of C5 by the immune complex EAC1423. If such inhibitory activity were less on PNH than on normal red cells, this might account for the increased fixation of C3 on PNH cells upon activation of complement. This, in turn, would partly account for their increased susceptibility to lysis by complement.

The purpose of this investigation was to examine the ability of membrane-derived complement inhibitory factors to interfere with hemolysis of PNH cells, and to present evidence for the existence of the same inhibitory activity in stromal extracts from normal and PNH red cells.

MATERIALS AND METHODS

Red Cells

Human and sheep red blood cells were collected, stored, and prepared for use as outlined by Rosse and Dacie. The standard suspension of human red cells (E) used in these studies contained 2.2 x 10⁸ cells/ml. The standard suspension of sheep red cells (E₈) contained 10⁸ cells/ml.

Antibodies

A cold-reactive anti-1 (anti-Step.), kindly supplied by Dr. and Mrs. John Crookston, Toronto, Canada, was used. Rabbit antibody to the boiled stromata of sheep red cells was diluted 1/100 and stored at −20°C. It was further diluted 1/400 on the day of use.

Complement and Complement Components

Human and guinea pig complement were obtained, stored, and prepared for use according to the method outlined by Rosse and Dacie. Partially purified C1 and partially purified C2 were prepared from guinea pig serum by the method of Borsos et al.

Buffers

An isotonic barbital (Veronal) saline buffer (VBS) containing 0.00015 M CaCl₂, and 0.0005 M MgCl₂, and 0.1% gelatin, prepared as described by Mayer, was used throughout unless otherwise indicated. One-tenth molar ethylenediaminetetraacetate (EDTA) was prepared as outlined by Frank et al. Isotonic sucrose buffer was made by dissolving 48.6 g of sucrose in 500 ml of H₂O. The pH was adjusted to 7.4 with 2N HCl. Calcium and magnesium ions and gelatin were added at the same concentration as in VBS. A solution of 60% isotonic sucrose–40% VBS (called “60% sucrose”) was used.

Cobra Venom Factor

Partially purified cobra venom factor (CoF) was prepared according to Kabakci et al.

Complement Inhibitory Factors

An anticomplementary factor obtained from extraction of human erythrocyte stromata at high ionic strength, designated I-H, and a substance accelerating SAC142 decay, termed DAF, were prepared by Dr. Edward Hoffmann, Gainesville, Fla. These were dialyzed against 0.9% saline overnight at 4°C, stored at −20°C, and kept over ice while in use.
Differential Density Gradient Centrifugation

Ficoll-Hypaque solution was prepared by mixing one part 33.9% Hypaque (diatrizoate sodium) in aqueous solution and 2.4 parts 9% Ficoll aqueous solution (specific gravity 1.080). A suspension of cells was overlayed on this mixture, then centrifuged at 200 g at room temperature for 20 min.

Extraction of Complement Inhibitor at Low Ionic Strength

Crude extracts of anticomplementary substances, termed I-H, were derived from normal and PNH human red cell stromata at low ionic strength according to the directions given by Hoffmann, with the following modifications. Between washings of intact red cells, white cells were removed by density gradient centrifugation with Ficoll-Hypaque, specific gravity 1.080. In some cases, when the stromata obtained after washing in phosphate buffer were still tinged with hemoglobin, they were mixed with 10 parts 0.010 M NaHCO<sub>3</sub>, stirred over ice for 1 hr, and sedimented by centrifugation at 15,000 g for 30 min at 0°C. This was repeated until the supernatant fluid became colorless. Stromata were mixed with 1% sodium deoxycholate, incubated at 37°C for 15 min, centrifuged as above and dialyzed first against phosphate buffer for 48 hr, then against 0.9% saline overnight. Protein concentrations were determined according to the method of Bailey.

Separation of Populations of PNH Red Cells

Subpopulations of PNH cells were separated by cobra venom lysis, since the complement-sensitive PNH cells (PNH II and PNH III cells) are susceptible to lysis in this system. To a given amount of a standard concentration of PNH cells (2.2 × 10<sup>6</sup>/ml) were added equal amounts of CoF-activated serum (serum incubated in equal parts with partially purified cobra factor at 37°C for 2 hr), 0.1 M EDTA, and ABO-compatible human serum diluted in equal parts with VBS as a source of complement. The mixture was incubated at 37°C for 1 hr and centrifuged at 20,000 g at 0°C for 30 min to collect stromata and intact cells. The lysed (complement-sensitive PNH III) cells were separated from unlysed (complement-insensitive PNH I) intact red cells by differential density gradient centrifugation. The stromata were washed with 0.005 M sodium phosphate buffer, pH 7.5, solubilized with 1% DOC, centrifuged, and dialyzed as outlined above. The unlysed red cells were washed and treated in the same manner as normal cells for extraction of the inhibitory factor.

Hemolysis Inhibition Assays

To determine the activity of extracts from human red cells in inhibiting lysis by complement, sensitized sheep cells (E<sup>S</sup>A) were prepared by mixing equal amounts of a standard suspension of sheep cells (× 10<sup>7</sup>/ml) and rabbit antiserum against boiled stroma of sheep red cells. Guinea pig complement was used at a dilution (usually 1/125) causing about 75% lysis of the standard EA suspension in a 0.75-ml reaction mixture. One-tenth milliliter of complement was placed in a series of tubes and 0.1 ml of doubling dilutions of extract from human red cells, 0.1 ml of sheep E<sup>S</sup>A, and sufficient buffer to bring the total volume to 0.75 ml were added. After incubation at 37°C for 1 hr, 5.0 ml VBS was added, the tubes were centrifuged, and the optical density of the supernatant fluid at 412 nm was determined. After appropriate subtractions for color due to complement, inhibitor, and nonspecific lysis, the degree of hemolysis inhibition was calculated for each dilution of inhibitor.

PNH Tests

Complement lysis sensitivity (CLS) tests were done as outlined by Rosse and Dacie with some modifications. The total volume of the reaction mixture used was 0.75 ml. To study the anticomplementary effect of the inhibitory factors, a parallel series of tubes was set up with 0.05 ml of inhibitor substituting for 0.05 ml buffer in a total volume of 0.75 ml. Cobra venom lysis tests were modified from the method outlined by Kabakci et al. Human complement was used at a dilution causing partial lysis of the PNH III cells in a standard suspension of PNH cells in a 0.5 ml reaction mixture. To each of a series of tubes were added in sequence, 0.1 ml volumes of red cells, CoF-activated serum, 0.1 M EDTA, doubling dilutions of inhibitor, and human serum diluted 1 in 2. The tubes were incubated for 60 min at 37°C. Five milliliters of VBS were added, the tubes were centrifuged, and the optical density of the supernatant fluid at 412 nm was determined. After suitable corrections for color due to complement, inhibitor, C3 proactivator, and nonspecific lysis, the percentage inhibition of hemolysis for each dilution of inhibitor was calculated.

Acidified serum lysis tests were modified from Dacie and Lewis using a total reaction volume of 0.42 with 0.2 ml undiluted serum, 0.02 ml 50% cell suspension. To investigate the activity of inhibitory factors in this system, 0.20 ml of doubling dilutions of inhibitor was substituted for an equal volume of VBS in a parallel set of tubes. After appropriate subtractions for color due to complement, inhibitor, and nonspecific lysis, the amount of hemolysis inhibited was calculated.

Since the CLS test uses an excess amount of antibody with variable amounts of complement, the following experiment was designed to examine the inhibitory activity of human E extracts in varying amounts of antibody with fixed excess amounts of complement. Doubling dilutions of an initial dilution of 1/50 of anti-I antibody were prepared at 37°C. Equal volumes of a standard suspension of red cells (2.2 × 10<sup>6</sup>/ml) and antibody were mixed (EA). At 37°C, 0.1 ml of each serum diluted one-half, inhibitor, and EA were added with 0.2 ml VBS. A parallel series of tubes included 0.1 ml each of serum and EA with 0.3 ml VBS. For 100% lysis control, 0.1 ml of a one-half dilution of a standard suspension of red cells was added to 0.4 ml VBS and saponin. These were incubated at 0°C for 15 min, then at 37°C for 60 min; 5.0 ml VBS were added and the cells were sedimented by centrifugation. The optical density of the supernatant fluid at 412 nm was determined. After correction for color due to complement, inhibitor, and nonspecific lysis, the fraction of inhibition was calculated.

E<sup>8</sup>AC142 Decay Assay

To make EAC4<sup>17</sup> equal volumes of E<sup>A</sup> and C1 (1000 U/ml) were mixed and incubated at 37°C for 5 min, then cooled. Cooled guinea pig serum diluted 1/20 in 0.015 M EDTA (C-E-DTA) was added and the mixture was allowed to incubate at 0°C for 15 min. The cells were washed in 0.015 M EDTA, then in VBS, and resuspended in VBS for storage, or in 60% sucrose at the time of use. The number of sites in the state SAC142 and the T<sub>max</sub> (time at which the largest number of such sites were present) were estimated using partially purified C2 and C-E-DTA. For the inhibition assay, a standard concentration of sheep EAC4 (1.65 × 10<sup>7</sup>/ml) was incubated with 1000 U of C1 at 37°C for 5 min. The cells were washed and standardized to the original concentration in 60% sucrose-VBS. Equal volumes of warmed EAC14 and a 1/2000 dilution of C2 in 60% sucrose-VBS were mixed at 30°C for T<sub>max</sub> time. The cells were washed and resuspended in 60% sucrose to...
the original concentration. Equal volumes of warmed EAC142 and inhibitor were mixed at 30°C. At appropriate time intervals, 1-ml volumes were withdrawn and added to 5 ml of guinea pig serum diluted 1/50 in 0.015 M EDTA. The tubes were incubated at 37°C for 60 min, centrifuged, and the optical density of the supernatant fluid at 412 nm was determined. In a control assay of SAC142 decay, VBS was substituted for the inhibitor, and subsequent procedures were carried out as above.

RESULTS

Effects of Complement Inhibitory Factors on PNH Cells

The lysis of normal and PNH cells of both subpopulations (the nearly normal PNH I cells and the markedly abnormal PNH III cells) in the complement lysis sensitivity test was markedly reduced or abolished by membrane-derived inhibitory activity from normal red cells (Fig. 1). In this test, antibody is present in relative excess and lysis is limited by the concentration of complement. When complement is present in relative excess and the concentration of antibody limits lysis, membrane-derived inhibitors completely inhibit lysis of both normal and PNH cells (Fig. 2 A and B).

To determine the relative efficiency of inhibition of lysis of normal and PNH red cells by limiting amounts of inhibitor, the amount of serum as a source of complement necessary to lyse 50% of the PNH III and of normal cells, respectively, was determined. A relative excess of antibody, this concentration of serum as a source of C′, and varying concentrations of inhibitor were mixed with the respective cells and the degree of inhibition determined. The lysis of normal and PNH cells was inhibited to the same relative degree by a given concentration of inhibitor (Fig. 3).

When the activation of complement is mediated...
through the alternative pathway by the presence of cobra venom factor (CoF), the membrane-derived inhibitor from normal cells is relatively ineffective in inhibiting lysis of PNH cells (Fig. 4).

A Comparison of Membrane-Derived Inhibitors From Normal and PNH Cells

When extractions of complement inhibitor were made from PNH and from normal red cells, approximately equal total amounts of protein were derived from equal numbers of normal and PNH cells. The solutions of extract were adjusted to equal protein concentrations before assays of the inhibition of hemolysis of sensitized sheep cells were done. There was no significant difference in inhibitory activity of extracts from PNH and normal cells (data not shown). The complement-sensitive PNH III cells were quantitatively lysed with cobra venom factor and fresh serum. The stromata and residual (PNH I) red cells were recovered separately by differential density centrifugation on a Ficoll-Hypaque gradient. The membranes of both populations were extracted, and the ability of the extract to inhibit the lysis of sensitized sheep red cells by guinea pig complement was compared to a similar extract from normal cells. The same degree of inhibition was obtained for an equal protein concentration of inhibitors from all three cell types (Fig. 5).

The effect of the same concentration of membrane extract for normal and PNH cells (70% PNH III and 30% PNH I) on the rate of decay of EAC42 is shown in Fig. 6. No significant difference was seen in the degree of increased degradation of this complex engendered by extracts for normal and PNH cells.

DISCUSSION

Hoffmann9 demonstrated the presence of an inhibitor of complement extractable from the membrane of the normal human erythrocyte. This inhibition is due to either an increased instability of the C42 complex or, perhaps, to some further interference with the ability of this complex to mediate

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**Fig. 4.** Inhibition by membrane-derived inhibitor of lysis of PNH cells by complement activated by antibody (closed symbols) and cobra venom factor (open symbols).

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**Fig. 5.** Inhibition of lysis of sensitized sheep cells by equivalent concentrations of extracts of normal (open symbols) and PNH III (closed symbols) red cell membranes. The PNH III cells were separated by differential lysis by cobra venom factor and complement.

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**Fig. 6.** The acceleration of decay of EAC42 on sheep cells mediated by extracts of normal (□) and PNH (■) membranes. The rate of decay in the absence of any membrane extract is also depicted (▲).
activation of C3 and/or C5. If this inhibitory activity were decreased or ineffective on the PNH cells that are susceptible to the lytic action of complement, this might explain the dramatic increase in the fixation of C3 to these cells when complement is activated.4

These studies have shown that the extraction of normal and PNH cells yields equal quantities of membrane-associated inhibitor of complement. This inhibitor from normal and PNH cells accelerated the decay of EAC\textsuperscript{1,4,2} equally. The same amount of inhibitor with the same activity is present in all the red cells examined. The fact that the same degree of inhibition of lysis of PNH and normal cells by the same amount of inhibitor is observed indicates that differences in membrane structure characteristic of PNH III cells do not modify the ability of the inhibitor to act. These data are consonant with the previous finding that the half-time of decay of the C42 on normal cells is the same as that on PNH cells.2 All these facts indicate that the increased fixation of C3 on PNH cells is not due to decreased concentration or activity of the membrane inhibitor, which would result in increased stability of the C42 complex. Other reasons for the increased C3 fixation, including increased efficiency of the alternative pathway or increased concentration of membrane component(s) capable of fixing the active fragment of C3/C3b must be sought.

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