Effect of Heparin on Complement Activation and Lysis of Paroxysmal Nocturnal Hemoglobinuria (PNH) Red Cells

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The effect of heparin upon the binding of the third component of complement (C3) to PNH red cells in vitro and their subsequent hemolysis is described. Heparin, in increasing concentrations, progressively inhibits membrane C3 fixation and hemolysis when the classic complement pathway is activated by anti-red cell antibodies. Heparin has a biphasic effect upon membrane C3 fixation and hemolysis when complement is activated in serum at decreased ionic strength (sucrose lysis) or in serum at decreased pH (Ham test). Heparin in concentrations above 2 U/ml inhibits C3 binding and hemolysis while lower concentrations of heparin enhance the consequences of complement activation by these two procedures. This enhanced complement activation may explain the increased hemolysis sometimes reported in PNH patients treated with heparin, and suggests that heparin may aggravate the consequences of pathologic alternative pathway complement activation in other diseases.

Erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH) are inordinately sensitive to lysis when complement is activated either in vitro or in vivo. Intravascular hemolysis of this complement-sensitive population of cells produces the major manifestations of this disease. Clinical observations of the effect of administration of heparin to patients with PNH are confusing. PNH patients have recurrent venous thromboses and hemolytic anemia induced by complement activation, but heparin, a known inhibitor of complement in vitro, appears to have a variable effect upon hemolysis.

Crosby and Dameshek initially described increased hemolysis when heparin was administered to a patient with PNH. Subsequently, other authors have described a variety of effects, including increased hemolysis, decreased hemolysis, or no change in hemolytic rate with heparin administration to PNH patients. There are no firm conclusions regarding the effect of heparin upon the hemolytic rate in patients with PNH. Crosby and Dameshek feel that the effect of heparin upon hemolysis in PNH patients is the result of activation of hemolytic enzymes by heparin at low concentrations, while at higher concentrations, heparin inhibits hemolysis. In order to clarify the effects of heparin upon complement activation and to understand better its effects in patients with PNH, we have reexamined the interaction between heparin and comple-
ment-mediated PNH red cell lysis in vitro utilizing newer techniques of complement investigation.

PNH red cells are detected by their increased sensitivity to lysis by complement when it is activated by a variety of procedures, including anti-red cell antibodies, incubation in serum at low ionic strength (sucrose lysis), or incubation in serum at decreased pH (Ham or acid serum lysis test). Complement is added to the membranes of both normal and PNH red cells by anti-red cell antibody and sucrose lysis complement activation, but much smaller quantities of membrane-bound complement as detected in the form of the third component of complement (C3) are required to lyse the PNH cells. Thus, consequences of complement activation upon PNH red cells may be studied both by determining hemolysis and by measuring the amount of C3 which is bound to these cells during the activation procedure. This paper describes the effect of heparin upon membrane C3 binding and PNH cell lysis by these complement activation procedures.

MATERIALS AND METHODS

Following are descriptions of the methods of measuring hemolysis and detecting membrane-bound C3 of PNH cells which have been exposed to complement-activating procedures in fresh human serum. These procedures are described in greater detail elsewhere. Initial human red cell concentrations have been adjusted by spectrophotometric determination of the optical density at 541 nm of red cell lysates, as described by Rosse and Dacie.

Activation by Anti-red Cell Antibodies

Whole blood from PNH patients was obtained by venipuncture and immediately placed into Alsever's solution until used. Red cells were washed three times in isotonic veranol-buffered saline with 1%, gelatin (VBS) and resuspended in VBS or the other buffers described below. Type-compatible sera obtained from normal donors were either used immediately or stored at -90°C until used. Anti-I and anti-i antibodies were obtained from patients with the chronic cold agglutinin syndrome as previously described (kindly provided by Dr. W. F. Rosse). A reaction mixture containing 0.1 ml of PNH red cells (2.2 x 10⁸/ml), 0.1 ml of anti-I or anti-i antisera diluted with VBS, 0.1 ml of compatible serum diluted 1 part in 10, and 0.1 ml of heparin (sterile heparin for injection, Pan Heparin, Abbott Laboratories, Chicago, Ill.) diluted in VBS was incubated at 4°C followed by a second incubation at 37°C for 30 min. All heparin concentrations were expressed as units per milliliter in the final mixtures. Five ml of VBS which contained 0.015 M EDTA (VBS-EDTA) were added and the mixture centrifuged at 12,000 g for 15 min. Lysis was determined by measuring the optical density of the supernatant at 412 nm. The resultant stroma was washed two additional times in VBS and resuspended in VBS for determination of membrane-bound C3.

Activation of Complement in Serum of Low Ionic Strength

A reaction mixture containing 0.1 ml of a 25% suspension of PNH red cells in isotonic veranol-buffered sucrose with added gelatin (VBSu) and 0.1 ml of normal serum undiluted or in a dilution of 1 part in 4 of VBSu, 0.1 ml of heparin diluted in VBSu, and 0.7 ml of VBSu was incubated at 37°C for 1 hr. Five ml of VBS-EDTA was then added, the mixture centrifuged, and lysis determined as described for the antibody-mediated procedure. The pellet was then washed once with VBS and once with distilled water. After two additional VBS washes, the cell stroma was resuspended to a concentration equivalent to 2.2 x 10⁸ red cells/ml. The volume of this final suspension was determined by dilution, lysis, and spectrophotometric determination at 541 nm of buffer controls which contained 0.1 ml of the 25% suspension used in the assay.
Activation of Complement in Acidified Serum

A reaction mixture containing 0.1 ml of a 25% suspension of PNH cells in VBS with a pH reduced to 6.4 by the addition of 0.15 M HCl (VBS-A), 0.1 ml of heparin diluted in VBS-A, and 0.9 ml of fresh, undiluted human serum which had been acidified to pH 6.4 with 1.0 M HCl was incubated at 37°C for 45 min. Five ml of VBS-EDTA were then added, the mixture centrifuged, and lysis determined as described above. The pellet was washed and resuspended as described for the sucrose activation procedure.

Detection of Membrane-bound C3

Human red cell membrane-bound C3 was measured by a modification of the method of Borsos and Leonard. This modification has been described previously. Surface C3 was quantitated by absorption of a known amount of rabbit anti-human C3 during incubation with red cell membranes. The amount of anti-C3 remaining in the supernatant was measured by lysis of C3-coated sheep erythrocytes with an excess of guinea pig serum as a source of complement. Purified human C3, and anti-C3, and C3-coated sheep cells were prepared as previously described. Duplicate 0.1-ml serial threefold dilutions of erythrocyte stroma or dilutions of known concentrations of human C3 were mixed with 0.1 ml of anti-C3 for 30 min to allow the absorption of antisera. One-tenth ml of C3-coated sheep cells were then added and allowed to react for 30 min, followed by the addition of 0.2 ml of guinea pig serum diluted 1 part in 40. After a final 30-min incubation, 5 ml of VBS were added, the mixture centrifuged, and the percentage of sheep cells lysed was determined by spectrophotometric measurement of free hemoglobin at 412 nm.

The degree of absorption of antibodies by C3-coated human red cell stroma was measured by the diminution of lysis of the C3-coated sheep cells. The diminution in lysis was compared to that engendered by graded quantities of fluid-phase C3. Such a standard curve was done as a part of each quantitative C3 assay. Due to possible differences between the antigens present in membrane-bound C3 and those in native C3, these measurements of membrane-bound C3 may be relative and not absolute. The pellet-washing procedure, after the complement reaction described above, recovered stroma of both complement-lysed and complement-unlysed red cells. Membrane-bound C3 was calculated from results obtained by analysis of duplicate serial dilutions of erythrocyte stroma.

RESULTS

Antibody Activation

Results from a complement activation experiment, using anti-I antisera at a dilution of 1 to 40 with varying amounts of heparin, are shown in Fig. 1. The PNH cells used in this assay were 60%, complement sensitive. The percentage

Fig. 1. Effect of heparin upon membrane binding of C3 and PNH cell lysis when anti-I sensitized cells are reacted with normal serum as described in the text.
of cells lysed and the amount of C3 on red cell stroma is shown in relation to the amount of heparin present in the reaction mixture. The amount of C3 is expressed as nanograms per stroma of $2.2 \times 10^8$ red cells; that is, the amount of C3 detected in 0.1 ml of a stroma suspension representing $2.2 \times 10^8$ red cells/ml. Progressive inhibition of C3 binding and hemolysis was noted for concentrations of heparin from 0.5 to 65 U/ml. Throughout this range, inhibition of hemolysis was proportional to inhibition of membrane binding of C3. The experiment depicted in Fig. 1 was repeated on three other occasions, once with red cells from a second PNH patient, with the same results.

Figure 2 shows the effects of heparin upon the lysis of PNH cells by anti-i antisera diluted 1 part in 5. Again heparin is seen to inhibit membrane binding of C3 progressively and, thus, to inhibit PNH cell lysis. This experiment has been repeated on two other occasions with similar results.

**Complement Activation in Serum at Low Ionic Strength**

Figure 3 shows the results of measurement of membrane-bound C3 and hemolysis when heparin was incubated with the sucrose lysis procedure. In contrast to the results obtained with antibody-mediated lysis, a more complex relationship is apparent. Membrane binding of C3 and hemolysis were inhibited by heparin concentrations above 2 U/ml, but for concentrations between 0.2 and 2.0 U/ml, red cell bound C3 and percentage hemolysis exceeded that for
cells which were not incubated with heparin. This experiment has been repeated on four other occasions, twice with red cells from a second PNH patient. Each time, lower concentrations of heparin have caused increased membrane binding of C3 and hemolysis.

To study further this low-concentration heparin enhancement, membrane-bound C3 was measured with varying serum concentrations along with constant amounts of heparin in the sucrose lysis procedure. Figure 4 shows the results of such a study with measurements in the absence of heparin compared to those observed with 1.25 U/ml and 5 U/ml of heparin. Low-concentration heparin enhancement of C3 binding was most evident with higher serum concentrations.

The addition of heparin in concentrations of 0.1 - 100 U/ml to a mixture of PNH red cells and fresh unacidified human serum did not cause lysis, and C3 was not bound to the red cells. Thus, heparin alone did not cause complement activation as detected by interaction with PNH red cells.

**Complement Activation in Serum at Decreased pH**

Figure 5 shows the results of determinations of membrane-bound C3 and hemolysis when heparin was added to the acidified serum test. The results are similar to those observed with the low ionic strength procedure; that is, in-
hibition of membrane binding of C3, with proportional inhibition of hemolysis, was observed at higher heparin concentrations. Lower heparin concentrations, however, enhanced membrane C3 binding and hemolysis when compared to that found in the absence of heparin. Similar results have been obtained in two additional acidification experiments.

DISCUSSION

Complement activation may occur by either a classic or an alternative pathway. In the classic pathway, antigen-antibody interaction leads to the sequential activation of the first, fourth, second, and third complement components. In the alternative pathway another series of proteins which have been less well defined may be activated by a variety of substances, including zymosan, bacterial toxins, and inulin. Ultimately, alternative pathway activation proceeds by activation of C3 proactivator and the subsequent cleavage of C3. Both pathways appear to share the final common reactions of the third through the ninth components.

We have previously shown that some PNH red cell lysis procedures act by the classic complement pathway, while others involve the alternative pathway. Lysis of PNH red cells in vitro by cold reactive antibodies occurs through activation of the classic complement pathway. Lysis of PNH red cells by incubation in serum at reduced pH occurs exclusively by the alternative pathway. The pathway of complement activation in serum at low ionic strength is unclear. Jenkins et al. and Bryant and Jenkins have shown that at moderately low serum concentrations complement activation by the sucrose lysis procedure occurs predominately by the classic pathway. On the other hand, we have previously found that at higher serum concentrations this form of complement activation occurs through both the classic and alternative pathways. Our previous studies would confirm that in dilute serum sucrose activation is primarily dependent upon classic pathway complement activation. By determining the effect of heparin upon these in vitro procedures, we hope to separate effects due to inhibition of either or both pathways.

When PNH red cells are lysed by these complement-activation procedures, the third component of complement is bound to the cell membrane. Quantitation of this membrane-bound C3 proves to be an accurate indicator of the amount of complement sequences initiated. Detection of this membrane-bound complement protein is useful in determining if differences in complement lysis are due to differences in activation of the early components of the complement cascade.

Heparin in high concentration inhibits complement lysis of PNH red cells by causing decreased binding of C3 to the membrane in all activation procedures. That is, detectable inhibition of hemolysis is always associated with decreased membrane-bound C3 on cells from heparin-containing reactions as compared to those of reactions without heparin. Others have suggested that heparin is inhibitory to activation of the terminal components, C5 to C9, but we are unable to verify these findings by the study of PNH cell lysis.

Previous investigators have shown that heparin inhibits complement by reacting with the first component. Our findings would support this contention in that antibody-mediated C3 binding was progressively inhibited by increasing concentrations of heparin. Since our studies have been done in the milieu of
whole human serum, we are unable to identify further which of the components of the classic pathway are affected. Rent and her colleagues have described a range of concentrations of heparin for which enhanced fourth and second component reactivity are present. The study of PNH cells described here fails to show a range of heparin enhancement of classic complement pathway action.

The effect of heparin upon PNH cell lysis by alternative pathway activation proves to be considerably more complex. In high concentrations, heparin inhibits C3 binding when red cells are incubated in acidified serum. Thus, heparin in high concentration inhibits alternative pathway complement activation. These studies do not allow speculation as to which reactions of this pathway are inhibited. Low concentrations of heparin increase PNH red cell-bound C3 when complement is activated in acidified serum or in serum at decreased ionic strength. Certainly the former of these procedures acts by alternative pathway complement activation only. Thus, from these studies heparin appears to have a biphasic effect upon alternative pathway complement activation, enhancing reactions at lower concentrations and inhibiting at high concentrations. These in vitro observations may explain the variable effect of heparin upon the rate of hemolysis in patients with PNH.

Activation of complement either by the classic or alternative pathway is involved in the pathophysiology of diseases other than PNH. A variety of immune hemolytic anemias, systemic lupus erythematosus, gram-negative sepsis, and some forms of glomerulonephritis are pathologic states in which increased complement activation is present. An effective inhibitor of complement activation would be of use in the treatment of these diseases.

The use of heparin as an inhibitor of complement in vivo was suggested when its first anticomplementary effect in vitro was noted. Ecker and Gross, in 1929, described the inhibitory effect of heparin upon sensitized sheep cell lysis in guinea pig serum. They were unsuccessful in decreasing the hemolytic ability of serum when the guinea pig was injected with intracardiac heparin. Subsequent reports of attempts to reduce the effects of complement activation by injection of heparin have been generally conflicting. In 1949, Owren successfully treated with heparin a patient who had severe hemolytic anemia. Others have described occasional dramatic beneficial effects of heparin in patients with autoimmune hemolytic anemia. Alternatively, Pirofsky has described several unsuccessful trials of heparin therapy of autoimmune hemolytic anemia. He and others tended to agree, however, that with case selection of appropriate patients, this form of therapy merited further clinical study. The observations described here may be helpful in this selection process.

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REFERENCES
HEPARIN AND C3 BINDING


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GL Logue