Complement activation on red cells by heparin-protamine complexes was studied by using whole human serum. C3 bound to red cells was measured by radiolabeled monoclonal antibody to C3, and fluid-phase C5a was determined by radioimmunoassay. Heparin and protamine in clinically relevant concentrations caused the binding of C3 to red cell membranes, and the measurement of C3 binding provided a sensitive indicator of complement activation produced by these complexes. Complement activation by these reagents occurred at concentration ratios of protamine and heparin at which protamine neutralized the anticoagulant effect of heparin. Heparin-protamine complexes appeared to bind to red cells and produce complement activation by the classic pathway. C5a generation with heparin-protamine complexes in serum was greatly enhanced in the presence of red cells and increased with increasing red cell concentration. This enhancement of complement activation in the presence of red cells was also seen as measured by depletion of available C3 hemolytic complement units in the fluid phase. Thus red cells seem to play an important role in activation of complement by heparin-protamine complexes.

To study complement activation by heparin-protamine complexes in the presence of physiological inhibitors and investigate the role a biologic membrane might play in such nonimmune-mediated complement activation, we examined the interaction of heparin, protamine, and red cells in whole serum. We found that heparin-protamine complexes bound to red cells and that C3 was deposited on red cell membranes in vitro when incubated with heparin and protamine. In addition, red cells markedly enhanced C5a generation and C3 depletion when complement was activated by these agents in serum. Thus, this nonimmunologic activation of complement is much more efficient when it occurs on the cell membrane than when it occurs in the fluid phase.

METHODS

Porcine intestine heparin (1,000 USP Pharmacopeia U/mL) was obtained from Elkins-Sinn Inc, Cherry Hill, NJ. Beef lung heparin (1,000 USP U/mL) was obtained from Upjohn Co, Kalamazoo, MI. Protamine sulfate (10 mg/mL) was purchased from Eli Lilly & Co., Indianapolis. Dilutions of heparin and protamine were made in veronal-buffered saline, pH 7.4, with 0.15 mmol/L CaCl₂ and 0.5 mmol/L MgCl₂ (VBS-M). For some experiments veronal-buffered saline without mixed metals (VBS) was used. EDTA was purchased from Haskan-Ladd Corp., Philadelphia. Ethylene glycol tetraacetic acid (EGTA) was purchased from Sigma Chemical Co., St Louis. N-butyl phthalate and bis-(2-ethylhexyl) phthalate were obtained from Fisher Scientific Co., Rochester, NY and mixed in a ratio of 6:4 (vol/vol). The activated partial thromboplastin time was determined by using reagents from American Dade, Miami.

Serum was used as a source of complement and fluid-phase complement inhibitors and was obtained from healthy AB blood group volunteer donors. The serum was either used fresh or stored at −70°C and thawed before use. Red cells were obtained from healthy volunteers in EDTA-containing tubes, washed five times in VBS-M, and resuspended in VBS-M before use. For some experiments, Selectogen cells having a specified panel of antigens (Ortho Diagnostic Systems, Raritan, NJ) were used. Monoclonal mouse IgG antibody against purified human C3 (anti-C3) was prepared from mouse ascites fluid (Bethesda Research Laboratories, Inc, Gaithersburg, MD) as described before. This antibody reacts with C3, C3b, C3bi, and C3c, but not C3d. The antibody was radiolabeled by using chloramine-T and brought to a concentration of 3 ng/mL. C3 Hemolytic Assay Kit (test tube method) was obtained from Diamedex Corp., Miami.

Measurement of C3 deposition on red cells. In a typical experiment, 100 μL red cells (5% by vol) were incubated for 30 minutes at

From www.bloodjournal.org by guest on November 15, 2017. For personal use only.
COMPLEMENT ACTIVATION BY HEPARIN-PROTAMINE

37°C with 200 μL heparin solution and 200 μL protamine solution (first incubation). After this incubation, 100 μL human serum was added and the incubation continued for another 30 minutes at 37°C (second incubation). The reaction was stopped by ice-bath temperature and rapid dilution with buffer. The reacted red cells were washed three times with VBS-M and resuspended in 200 μL VBS-M. An aliquot was removed for the determination of cell number by an automated blood cell counter (Coulter Electronics, Inc., Hialeah, FL). The remaining cells were assayed in triplicate for the presence of surface-bound C3. Fifty microliters of reacted cells was added to 50 μL radiolabeled anti-C3 that had been layered over 100 μL phthalate oils in 400-μL microfuge tubes (Analytic Laboratory Accessories, Rockville Centre, NY), and the cells were incubated for 20 minutes at 37°C. Efficient separation of cell-bound from unbound anti-C3 that remained in aqueous phase was accomplished by centrifugation of the cells through the oil mixture in a Beckman Microfuge (Fullerton, CA) for three minutes. The cell pellet was removed by excision of the tube tip with a razor blade, and its radioactivity was quantified by a gamma scintillation counter (TM Analytic, Elk Grove, IL). Results were expressed as femtograms anti-C3 bound per cell. Nonspecific binding of anti-C3 to red cells incubated with buffer for 30 minutes at 37°C was deducted in the final calculation unless specified and was approximately 0.2 fg/cell.

Measurement of C5a generation. For these experiments, 10 μL heparin solution and 10 μL protamine solution or 20 μL VBS-M were incubated with 50 μL red cell suspension or buffer for 30 minutes at 37°C followed by the addition of 230 μL fresh serum. The second incubation was carried on for another 30 minutes. The mixture was then centrifuged and the supernatants removed for assay of fluid-phase C5a desArg. C5a desArg was measured by radioimmunoassay (Upjohn Diagnostics, Kalamazoo, MI). The absolute values of C5a desArg were extrapolated from standard curves and expressed as nanograms per milliliter. Background C5a detected in the absence of heparin and protamine was approximately 250 ng/mL and was deducted in the final calculation.

RESULTS

Heparin and protamine complexes activate complement as measured by cell-bound C3. Figure 1 shows the results of a set of experiments in which the protamine concentration was held constant at 10 μg/mL and varying concentrations of heparin added. After 30 minutes' incubation with serum, C3 bound to red cells was maximal at approximately 0.75 U/mL heparin. This concentration of heparin was approximately the amount that was neutralized by the amount of protamine present when the mixture was assayed for ability to prolong the activated partial thromboplastin time. In other experiments (results not shown), the final heparin concentration was held constant at 1 U/mL, and the protamine concentration was varied. Maximal C3 binding occurred at protamine concentrations of between 7.5 and 10 μg/mL. C3 binding to red cells was not affected by the source of heparin (ie, beef lung or porcine intestine source) or by the antigenic makeup of the red cells, including Rhnull cells.

When heparin-protamine and serum were incubated in the absence of red cells for five and 15 minutes and then red cells added and incubation continued for an additional 30 minutes, the amount of C3 bound per cell was similar to cells incubated in heparin-protamine and serum without preincubation. In most of the experiments described in this text, protamine was added last. However, varying the sequence of addition of heparin, protamine, or red cells in the first incubation did not alter the amount of C3 binding to red cells at the concentrations of heparin-protamine used in these experiments.

The effect of varying the duration of the second 37°C incubation is shown in Fig 2. At the times noted, the incubation was terminated in separate tubes, the cells washed, and bound C3 measured. Maximal C3 binding occurred at five minutes. With longer incubations, the quantity of cell-bound C3 fell due either to degradation of C3b to C3dg or dissociation of complexed C3 from the cell.

The relationship between concentration of heparin-protamine complexes and red cell C3 binding is shown in Fig 3. In this experiment, the ratio of heparin to protamine present in the red cell preincubation was held constant at neutralizing concentrations, but the total amount of both chemicals was varied. For these experiments the heparin- and protamine-treated cells were incubated with 100 μL of undiluted human serum for five minutes to measure the maximum detectable C3 levels. As shown, an increase in the concentration of the heparin-protamine mixture up to 17 units of heparin and 170 μg of protamine led to increased cell-bound C3.
their original volume with either VBS-M, VBS with MgEGTA (15 mmol/L EGTA with 7.5 mmol/L Mg²⁺), or VBS with EDTA (15 mmol/L). This concentration of MgEGTA inhibited the classical pathway complement activation on red cells when induced by anti-I antibody but produced less than 10% inhibition of the alternative pathway complement activation when induced by zymosan. The results of incubation of heparin-protamine-treated red cells with these reagents is shown in Fig 4. In this figure, the amount of C3 bound to cells in the absence of heparin and protamine is shown in comparison to the amount of C3 binding that occurred with heparin and protamine in the presence of these chelating agents. Both Mg-EGTA and EDTA completely eliminated C3 binding produced by heparin and protamine in the presence of serum. Thus, the C3 binding was produced entirely by the classical complement pathway.

Figure 5 shows the effect of varying concentrations of heparin-protamine complexes on fluid-phase C5a in the presence and absence of red cells. As shown in this figure, C5a generation was minimally elevated at 1 U/mL heparin and 10 μg/mL protamine in the presence and in the absence of red cells. With heparin and protamine concentrations of 5 U/mL and 50 μg/mL, and 10 U/mL and 100 μg/mL, respectively, there was a marked elevation of C5a desArg levels in the presence of red cells, whereas they were barely detectable above the background in their absence. This experiment clearly showed that complement activation occurring on the cell membrane was much more effective in generating the potent anaphylotoxin C5a than that occurring in the fluid phase.

Figure 6 shows similar experiments at the same serum concentration but with the heparin and protamine concentrations remaining constant at 5 U/mL and 50 μg/mL, respectively, whereas the final concentration of red cells was varied. 
As shown in the figure, the C5a desArg concentration increased linearly as the red cell concentration increased, again showing the importance of the cell membrane in propagating the complement activation produced by heparin-protamine complexes.

The effect of red cells on complement activation by heparin-protamine complexes was also studied by measuring depletion of fluid-phase hemolytic C3. In these experiments 200 μL red cells (20% by vol) or 200 μL buffer was incubated with 400 μL heparin (final concentration, 4 U/mL) and 400 μL protamine (final concentration, 40 μg/mL) or 800 μL buffer for 30 minutes at 37°C. Two hundred microliters of serum was added and 37°C incubation carried on for another hour. At the end of this incubation, the tubes were centrifuged and the supernatants aspirated for C3 hemolytic cells.

The C5a desArg concentration was found to increase as the red cell concentration increased, again showing the importance of the cell membrane in propagating the complement activation produced by heparin-protamine complexes.

DISCUSSION

Heparin-protamine mixtures activate complement by the classical pathway. This activation leads to deposition of the third component of complement present in whole serum to red cells in the reaction mixture. Measurement of C3 bound to cells provides a sensitive indicator of this reaction. Using this indicator we were able to accurately study heparin-protamine reactions. The optimal complement-activating concentrations of heparin and protamine appear to be the same as those required for neutralizing the anticoagulant effect of heparin. We were also able to detect complement activation with a considerably smaller concentration of the heparin-protamine mixture than previously described, i.e., with therapeutically achievable concentrations of these two reagents.

Rent et al. in their experiments using CH5O assays showed that 50% of complement was consumed with 4 U/mL heparin and 40 μg/mL protamine. With a 50% reduction in the concentration of heparin and protamine, 10% of the complement was consumed. When using 8 U/mL heparin and 80 μg/mL protamine, there was maximal depletion of complement activity, which persisted with higher concentrations of heparin and protamine. Our assay system, which used detection of C3 bound to red cell membranes, was more sensitive at both ends of the spectrum. With as little as 0.5 U/mL heparin and 5 μg/mL protamine, we could detect significant C3 binding to red cells. This C3 binding continued to increase as the concentrations of heparin and protamine were increased. We were also able to confirm previous observations that heparin and protamine activated complement by the classical pathway and in this manner behaved like antigen-antibody complexes. We also found that red cells interacted with the heparin-protamine mixture. Our washing experiments demonstrated binding of the complement-activating ability when cells were preincubated with heparin and protamine without serum. This complement-activating activity did not occur when the cells were incubated with either of the reactants alone and were subjected to a washing step before the other was added. This red cell—binding activity did not depend on minor blood groups antigens and was not removed or enhanced by enzyme treatment of the red cells.

Al-Mondhiry et al., who used radiiodinated protamine, found that the protamine bound to membranes of circulating cells and the cell-bound radioactivity was increased when both heparin and protamine were present in the incubation mixture. In their experiments, the cell-bound radioactivity could be reduced but not totally eliminated by repeated
washing of labeled cells with buffer. In experiments with red cells that were washed after being incubated in heparin-protamine mixtures, we indirectly demonstrated their presence on red cells by their ability to activate complement. Protamine alone, even if bound to red cells, did not activate complement in our series of experiments. The ability of heparin-protamine complexes to bind to red cell membranes and activate complement might be an important mechanism of complement activation by these agents.

The sheer number of circulating red cells would make them ideal candidates for heparin-protamine complex binding and subsequent circulation of cells able to activate complement. Our experiments showed that red cells markedly enhanced complement activation produced by heparin and protamine. The amount of C5a generated by heparin-protamine complexes was markedly increased when red cells were added. This enhancement was more marked with increased concentrations of red cells. The presence of an erythrocyte membrane may facilitate the assembly of C5b-8, which is required for the release of C5a. However, the increased depletion of available hemolytic C3 activity indicates that there is true enhancement of complement activation by heparin-protamine complexes in the presence of red cells. The reason for this enhanced complement activation may relate to the increased efficiency of C1 binding as well as the relative ineffectiveness of fluid-phase inhibitors. Caughman et al in their studies found that C1 inhibitor was 10,000-fold more effective on a weight basis at inhibiting C1 hemolytic activity when C1 was in the fluid phase than when it was bound to indicator cells. They further postulate that it is likely that the binding of C1 to indicator cells forces the C1 macromolecular complex to assume and maintain a conformation in which the interaction of C1 inhibitor with the proteolytic subunits is not as favored as it is when the C1 molecule exists in the fluid phase. The binding of heparin-protamine complexes to a cell membrane would contribute to the ease with which C1 is bound to the cell membrane and with which it remains relatively protected from the fluid-phase C1 inhibitor.

Heparin-protamine complexes are one of the several factors that can cause complement activation during cardiopulmonary bypass. Although this usually produces minimal clinical consequences, an occasional patient may have severe complications including hypotension and pulmonary leukocyte sequestration, pulmonary edema, and progressive hypoxemia. Pathological activation of the complement system with generation of potent anaphylatoxins has been strongly implicated in these patients. Clinical studies measuring parameters of complement activation during and after cardiopulmonary bypass have thus far failed to detect increased C5a levels in patients. A possible explanation forwarded by one group is that the excess C5a generated binds to the neutrophils and thus is not detected in plasma. It should be pointed out, however, that in this and other clinical trials no patient who actually developed postpump syndrome was studied.

It can be postulated from our experiments that, given a proper set of circumstances such as increased complement activation due to optimal concentrations of heparin and protamine along with reduced activity of regulatory factors for red cell-bound classical pathway C3 convertase and its components, pathophysiological complement activation may indeed occur. Potent anaphylatoxins would be generated during protamine neutralization of heparin after cardiopulmonary bypass. The rarity of such profound untoward interactions underscores the importance of several factors in the causation of this kind of untoward reaction.

ACKNOWLEDGMENT

The authors are indebted to Norma Hoffman and Barbara Mueller for secretarial support.

REFERENCES

Effect of RBCs on the activation of human complement by heparin-
protamine complexes

KA Shastri, MJ Phillips, S Raza, GL Logue and PK Rustagi