ACTIVATION OF THE ALTERNATE COMPLEMENT PATHWAY BY AUTOLOGOUS RED CELL STROMA

By THOMAS R. POSKITT, H. PHILIP FORTWENGLER, JR.,
AND BEVERLY J. LUNSKIS

(*From the U.S. Army Medical Research Laboratory, Fort Knox, Kentucky 40121*)

The syndrome of disseminated intravascular coagulation (DIC)[1] is a recognized complication of acute hemolytic episodes in both man (1–3) and laboratory animals (4, 5) and has been induced experimentally in monkeys and dogs by infusion of autologous red blood cell stroma (6, 7). The mechanism of stroma-induced DIC may be analogous to the demonstrable procoagulant activity of red cell membrane phospholipids in vitro. However, an additional or alternative chain of events involving activation of the complement system is suggested by the well-studied model of DIC, the Sanarelli-Shwartzman reaction, produced by two appropriately spaced intravenous injections of bacterial endotoxin. The recent work of Fong and Good (8) and Margaretten (9) indicate a requirement for platelets and an intact complement system for endotoxin-induced DIC. Complement activation by endotoxin proceeds primarily, if not entirely, via the recently described alternate pathway (10). In view of the ultrastructural similarity between endotoxin and red cell stroma (i.e., bilaminar membrane [11]) and their ability to produce DIC, we have initiated studies to determine whether red cell stroma is also capable of activating the alternate complement pathway and to attempt to relate this function to the sequence of events leading to DIC.

Materials and Methods

Preparation of Autologous Red Cell Stroma.—Hemoglobin-free red cell stroma was prepared from five human volunteers by a modification of a previously described method (7). 50 ml of anticoagulated whole blood was collected from each donor. The red cells were washed three times in cold 0.85% saline with aspiration of the buffy coat and upper 5% of red cells after each wash to minimize platelet and leukocyte contamination. Red cells were lysed by suspension in 50 volumes of 20 mosmol phosphate buffer with gentle swirling for 1 h. Ghosts were separated from supernatant hemoglobin by centrifugation at $7,000 \times g$ for 60 min, resuspended in 0.85% saline, and recentrifuged at $7,000 \times g$ for another 60 min. The supernatant was aspirated, and cold 0.85% saline was added equal to the volume of ghosts. The suspension

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was incubated for 12–16 h at 4°C to leach out remaining hemoglobin, and then centrifuged at 27,000 × g for 30 min. The ghosts were suspended in an equal volume of 0.85% saline and 1 ml aliquots were distributed to three 12 × 72 mm polypropylene test tubes. One tube was designated "autologous red cell ghosts." The second and third tubes, immersed in an ice water bath, were sonicated at 20,000 cps (Biosonik III, Bronwill Scientific, Rochester, N. Y.) for 1 min and 5 min and designated "autologous red cell stroma, 1 min" and "autologous red cell stroma, 5 min," respectively.

This same procedure with the following alterations was employed for the production of hemoglobin-containing RBC stroma. The supernatant hemoglobin solution produced by osmotic lysis was concentrated by negative pressure ultrafiltration and centrifuged at 40,000 × g for 15 min to remove all stromal material. An equal volume of this hemoglobin solution was added to the autologous ghosts before sonication in place of the 0.85% saline. The total amount of hemoglobin added was calculated to correspond to that which had been contained in the given volume of RBC ghosts.

Preparation of Autologous Platelet Stroma.—Human platelets were separated from 50 ml of anticoagulated whole blood on a Hypaque-Macrodex discontinuous gradient (12), washed four times in 0.85% saline, and sonicated for 5 min at 20,000 cps as described above. The whole sonicate was centrifuged at 60,000 × g for 30 min and the sediment, designated "autologous platelet stroma," resuspended in 0.85% saline and kept at 4°C for 16 h before use.

Incubation of Stroma with Autologous Serum.—0.02 ml of each stroma preparation was incubated with 0.1 ml of fresh autologous serum at 37°C for 60 min. Controls consisted of the following: 0.1 ml aliquots of serum containing (a) 0.02 ml of saline subjected to the same conditions (centrifugation, sonication, incubation) as the stroma preparations, (b) 0.02 ml of autologous platelet stroma, (c) 0.02 ml of intact autologous RBC's, and (d) 0.02 ml of concentrated hemoglobin, all incubated at 37°C for 1 h. As a positive control for activation of the alternate pathway, 0.02 ml of an inulin suspension (25 mg/ml in 0.5% saline) was incubated with serum for 1 h at 37°C. All samples were stored at −75°C until ready for assay.

Antiserum.—Antiserum to C3-Proactivator (C3PA) was purchased from Behring Diagnostics, Woodbury, N. Y.

Immunoelectrophoresis.—Immunoelectrophoresis was carried out in 1% agar in sodium barbital buffer, 0.05 M, pH 8.2, containing 0.01 M EDTA. 0.02 ml wells and 0.085 ml troughs were employed. The period of electrophoresis was 3 h at room temperature with a current of 5 mA per slide.

RESULTS

Immunoelectrophoresis.—Evidence for activation of the alternate complement pathway by autologous red cell stroma is presented in Figs. 1 and 2. In slide *A* of Fig. 1, the upper well contains serum incubated with inulin; the lower well contains control serum (serum plus saline). The trough contains antiserum to C3PA. The characteristic beta mobility of C3PA in the control and inulin-treated sample is evident. Activation of the alternate pathway with cleavage of C3PA to C3-Activator (C3A), represented by an additional precipitin arc with gamma mobility, is clearly seen in the inulin-treated serum. In slides *B*, *C*, and *D* the upper wells contain serum incubated with autologous RBC ghosts, 1 min stroma, and 5 min stroma, respectively. The generation of C3A from C3PA is clearly seen in all three samples. Slide *E* demonstrates the inability of concentrated autologous platelet stroma to effect the cleavage of C3PA, thus rendering it unlikely that contaminating platelet membranes were responsible
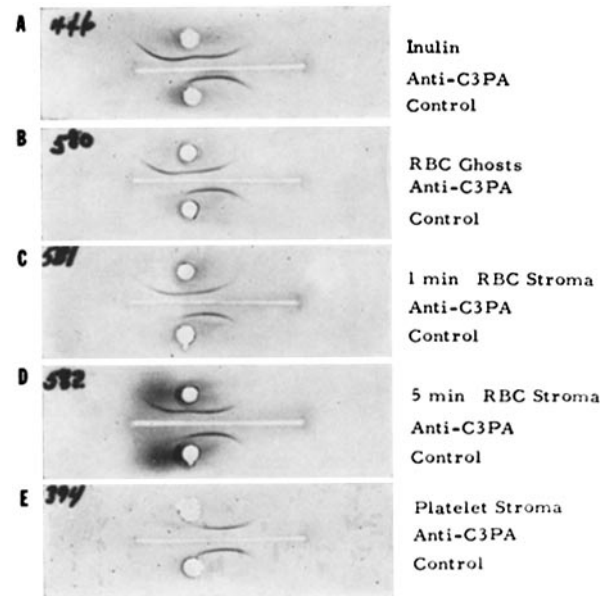
FIG. 1. Slides A–D demonstrate the generation of C3A from C3PA by inulin and autologous RBC membranes. Slide E demonstrates lack of this activity in concentrated platelet stroma. Anode is to the right, cathode to the left.

for the C3A generating activity of the RBC stroma preparations. Similarly, incubation of serum with intact autologous RBC's had no effect on C3PA.

Fig. 2 demonstrates the effect of hemoglobin on stromal activation of C3PA. In slides A, B, and C (corresponding to B, C, and D of Fig. 1), hemoglobin shows a mild potentiating effect on C3A generation. Slide D, in which concentrated hemoglobin was incubated with serum, demonstrates very slight generation of C3A. This activity was most likely the result of a small quantity of stroma material remaining after centrifugation since a second centrifugation at 40,000 × g for 15 min removed it completely. Although there was a small degree of variation among the five individuals tested, all samples of RBC ghosts and sonicated ghosts were capable of generating C3A. In no instance did platelet stroma or intact red cells produce this effect.

DISCUSSION

Acute hemolytic episodes in both man and laboratory animals are often accompanied by coagulation changes ranging from mild hypercoagulability (13, 14) to overt disseminated intravascular coagulation (4, 15, 16). DIC in humans has been associated with both immune (e.g., hemolytic transfusion reactions [1, 4]) and nonimmune (e.g., falciparum malaria [5]) hemolysis, suggesting that hemolysis per se rather than the hemolytic agent is the critical
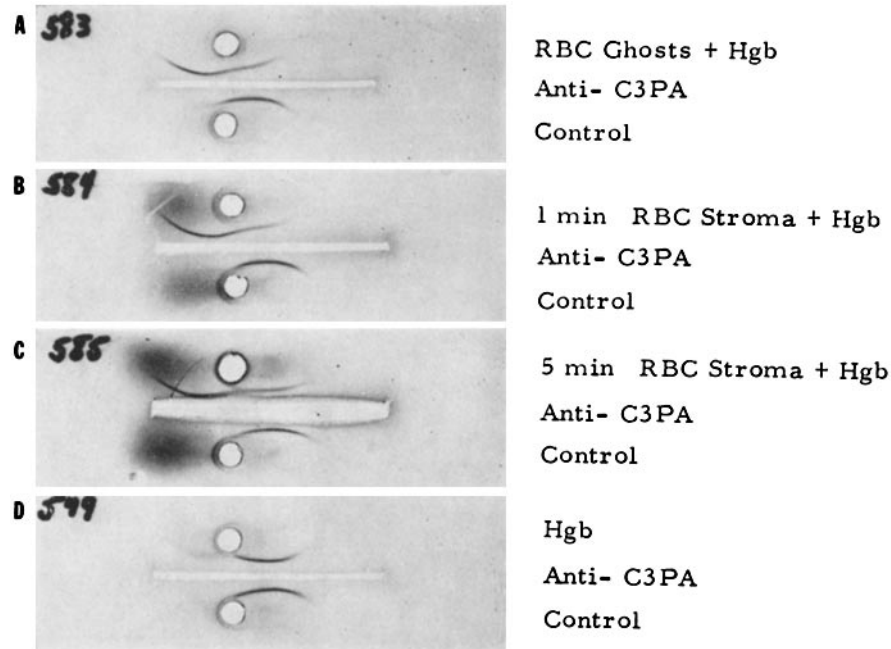
Fig. 2. Slides *A–C* demonstrate that hemoglobin is ineffective in inhibiting the RBC stroma activity. In slide *D*, stroma-free hemoglobin is incapable of generating C3A.

factor in DIC induction. Studies in laboratory animals have strongly supported these observations, especially with the development of a hemolytic transfusion reaction (HTR) model in the Cynomolgus monkey (*Macaca irus*) (2, 16). Hemolytic antibody, prepared by immunization of donor monkeys with allogeneic RBC's, when infused into recipient monkeys as either whole plasma or the hemolytic IgG fraction, produces brisk hemolysis followed by laboratory and histopathologic evidence of DIC. Similar results are obtained by infusion of incompatible RBC's into an isoimmune recipient (3), a situation analogous to the usual clinically encountered HTR.

In both these models, hemolysis is achieved by activation of the classical complement pathway by IgG antibody fixed to RBC membrane antigens. Consequently, the mechanism for initiation of DIC in these models is difficult to assess in view of the known procoagulant activity of RBC membranes as well as the reported ability of antigen-antibody complexes themselves to induce DIC (17, 18). However, the observation by Rabiner et al. (6) and Birndorf et al. (7) that autologous RBC stroma itself is capable of producing mild DIC when infused into dogs and monkeys simplifies the problem since a complement-fixing antigen-antibody system, in the form of hemolytic antibody, is no longer present. This observed effect of RBC stroma on the coagulation system, there-

fore, appeared to be due to the known in vitro procoagulant activity of membrane phospholipids and seemed to offer an explanation for the occurrence of DIC with acute hemolysis in man.

However, doubts concerning the ability of intravascular hemolysis per se to activate the coagulation system have been raised by Triantaphyllopoulos (19, 20), who was able to nullify the in vitro procoagulant effect of RBC stroma by addition of purified hemoglobin. Since hemoglobinemia is a natural consequence of massive intravascular hemolysis, Triantaphyllopoulos's findings appear to cast doubt on a direct activation of the coagulation system in vivo by RBC membrane phospholipids.

In the present study we have presented evidence for activation of the alternate complement pathway by autologous red cell membranes, which may provide an alternative mechanism for hemolysis-initiated DIC. RBC ghosts sonicated for 5 min at 20,000 cps, the form used by Birndorf et al. in their monkey model, effectively cleaved C3-Proactivator (C3PA) to C3-Activator (C3A). RBC ghosts sonicated for 1 min as well as unsonicated ghosts were equally effective in generating C3A. Intact RBC's, in contrast, failed to produce this effect. Moreover, the presence of free hemoglobin had no inhibitory effect on the stromal C3A generating activity, in contrast to its reported inhibitory effect on the in vitro procoagulant activity of RBC stroma.

It is possible that trace amounts of platelet phospholipid contaminating the infused autologous RBC stroma preparation could account for the coagulation changes observed by Birndorf et al. and Rabiner et al. However, the C3A generating activity of our RBC stroma preparations does not reside in a contaminating platelet fraction as evidenced by the total inactivity of concentrated platelet stroma.

The significance of our present finding that human autologous RBC stroma can activate the alternate complement pathway may lie in the recently recognized interrelationships between the complement and coagulation systems. Zimmerman et al. (21) have described prolongation of whole blood clotting times and decreased prothrombin consumption in a rabbit strain genetically deficient in C6. The addition of highly purified C6 to the deficient blood corrected the coagulation abnormality. Further work by these investigators (22) has demonstrated that known activators of the alternate pathway (endotoxin, staphylococcal protein A, inulin, immunoglobulin aggregates) are effective in accelerating clotting of normal rabbit blood but are ineffective in C6 deficient blood. Furthermore, Girando et al. (23) and Lee et al. (24) have reported that the thrombocytopenia and intravascular coagulation induced by infusion of thromboplastin into rabbits and mice is also dependent upon the complement system. These findings coupled with reports that substances like endotoxin cannot initiate DIC in animals rendered hypocomplementemic by cobra venom factor (8) demonstrate a functionally important interrelationship between the complement and coagulation systems.

The nature of the interaction between these two systems is ill-defined although evidence suggests that platelets may be the intermediary. The inability of endotoxin to produce DIC in rabbits rendered severely thrombocytopenic by antiplatelet antibody (9) as well as the ability of platelet factor 3 (PF-3) (25) and frozen-thawed platelets (26) to induce intravascular coagulation support this hypothesis. The interaction of complement with platelets in hemolysis-initiated DIC is currently under investigation in the Cynomolgus monkey model. Two proposed mechanisms for RBC stroma activation of the coagulation system are presented in Fig. 3—one involving direct activation by membrane phospholipids and the other requiring the interaction of activated complement components with platelets to effect the release of PF-3. Complement-dependent release of platelet constituents by soluble antigen-antibody complexes or particulate matter has been demonstrated in nonprimate species (27), and a similar process might occur in primates. In addition, recent studies
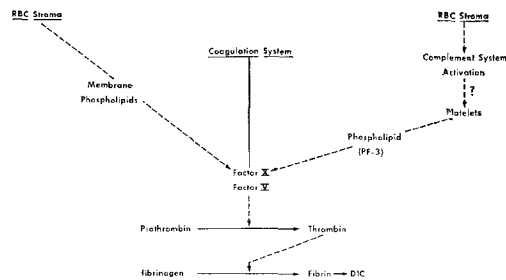

FIG. 3. Proposed mechanisms for the production of DIC by autologous red cell stroma.

by Siraganian and Alexander (28) have demonstrated that platelet lysis with release of PF-3 can be produced in vitro by activation of the alternate complement pathway. The possibility that complement activation by RBC stroma in the microenvironment of the platelet might lead to the release of PF-3 is an attractive hypothesis. However, it is necessary to repeat these RBC stroma studies in hypocomplementemic and thrombocytopenic monkeys in order to define the roles of the proposed intermediaries of DIC; and to assess the relative significance of RBC membrane activation of the coagulation system by direct (i.e., membrane phospholipids) and indirect (i.e., complement, platelets, PF-3) means.

SUMMARY

The present study demonstrates the ability of human autologous RBC stroma to activate the alternate complement pathway (C3-Activator system, properdin system), as evidenced by the generation of C3-Activator (C3A) from C3-Proactivator (C3PA) when RBC ghosts and sonicated ghosts are incubated with autologous serum. Intact RBC's, hemoglobin, and concen-

trated platelet stroma, on the other hand, are inactive in this regard. We postulate that this in vitro activity of RBC stroma may occur intravascularly when erythrocytes are damaged by immune or nonimmune mechanisms. The ensuing interaction of activated complement components with platelets leading to release of platelet factor three (PF-3) may constitute a mechanism for activation of the coagulation system during acute hemolytic episodes.

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