BLOOD COAGULATION INITIATION BY A COMPLEMENT-MEDIATED PATHWAY*

By THEODORE S. ZIMMERMAN,‡ M.D., AND
HANS J. MÜLLER-EBERHARD, M.D.

(*From the Department of Experimental Pathology, Scripps Clinic and Research
Foundation, La Jolla, California 92037*)

Bacterial endotoxins and antigen-antibody aggregates activate complement, accelerate blood coagulation, and are implicated in intravascular blood clotting (1–7). That their effect on blood coagulation proceeds through the activation of the complement system has been suspected but has never been shown. We have found that these and other complement-activating materials initiate blood coagulation through a complement-mediated pathway.

The demonstration of a coagulation abnormality in blood of a rabbit deficient in the sixth component of complement (C6) and its correction with purified C6 has provided direct evidence for the involvement of complement in normal hemostasis (8). The experiments reported here indicate that activation of complement can initiate blood coagulation and suggest an important role for complement in pathologic blood coagulation.

Materials and Methods

Inulin, chemically pure (Pfanstiehl Chemical Corp., Waukegan, Ill.), was dissolved at a concentration of 20 mg/ml in barbital-buffered saline (0.005 M sodium barbital, 0.145 M NaCl, 0.0005 M MgCl$_2$, 0.00015 M MgCl$_2$, pH 7.5) at 60°C. A precipitate formed when the solution was cooled to room temperature and was partially removed by centrifugation at 1000 g for 5–10 min in a Serofuge (Clay-Adams, Inc., New York). 0.1 ml aliquots of the 20 mg/ml solution were mixed with 1 ml of rabbit blood for determination of the effect on prothrombin consumption; 0.05 ml aliquots were used for determining the effect on clotting times.

Salmonella endotoxin, obtained from Dr. Otto Lüderitz of the Max-Planck-Institut für Immunbiologie, Freiburg, Germany, was dissolved in distilled water at a concentration of 2 mg/ml. 20 μl was mixed with 1 ml of blood. Staphylococcal protein A, obtained from Dr. Gunnemar Stålenheim, The Wallenberg Laboratory, Uppsala, Sweden, was dissolved at a concentration of 1.3 mg/ml. 0.1 ml aliquots were mixed with 1 ml of blood.

Gamma globulin (Cohn fraction II, a gift of Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.) was dissolved in barbital-buffered saline at a concentration of 10 mg/ml and subjected to centrifugation at 170,000 g (average) for 90 min in a SW-50 swinging bucket rotor in a Spinco L-2 ultracentrifuge (Beckman Instruments, Palo Alto, Calif.). The top third of the supernatant was removed and an aliquot heated at 60°C for 10 min. An unheated aliquot was used as the control. Aliquots of 0.2 ml were mixed with 1 ml of blood. Kaolin, NF (J.T. Baker Chemical Co., Philipsburg, N.J.) was suspended in barbital-buffered saline at a concentration of 20 mg/ml and was used in 0.1 ml aliquots for prothrombin consumption experiments and in 0.05 ml aliquots for clotting time experiments. Isolated rabbit and human C6 was prepared as previously described (9). It was used in 0.1 ml aliquots at a concentration of 25 μg/ml.

Rabbit blood was obtained from the hind leg vein using a No. 19 small vein infusion set (McGaw Laboratories Inc., Glendale, Calif.) and disposable polypropylene syringes (Sherwood Medical Industries Inc., Deland, Fla.). A two-syringe technique was used and great care was taken to avoid contamination with tissue juices. 1 ml aliquots of freshly drawn blood

TABLE I

Effect of Kaolin and Inulin on Clotting Times of Normal and C6-Deficient Rabbit Blood

Type of blood	Material added	Clotting time
		min
Normal	Kaolin	5–10
Normal	Inulin	20
Normal	Buffer	60
C6 deficient	Kaolin	5–10
C6 deficient	Inulin	230
C6 deficient	Buffer	220

* Clotting times were performed in polypropylene tubes at 25°C. Kaolin and inulin were used in similar quantities. For additional details see Materials and Methods.

were added immediately to duplicate 12 × 75-mm polypropylene tubes (Falcon Plastics, Oxnard, Calif.) containing the substance to be tested. Mixing was achieved by gentle tapping. Clotting times were determined at 25°C using a serial, three-tube technique (8). For prothrombin consumption determinations, tubes were incubated at 37°C for the indicated time. The clotting process was then stopped by removal of the tubes to an ice water bath and the addition of 0.04 ml of 0.5 M citrate buffer, pH 5.0. The serum was then assayed for residual prothrombin (8). Results are expressed as the average values from duplicate incubation mixtures.

RESULTS

Inulin, as compared to buffer, shortened clotting times (Table I) and markedly accelerated prothrombin consumption in normal rabbit blood. The latter effect was apparent after 10 min incubation at 37°C (see Table II). No such clot-promoting effect of inulin could be demonstrated in C6-deficient blood. Since prothrombin consumption is retarded in C6-deficient blood (8), we ex-

TABLE II

*The Acceleration of Prothrombin Consumption by Complement-Activating Materials**

Material tested	Type of blood	Incubation time	Prothrombin consumed
		min	%
Inulin	Normal rabbit	10	>99
Buffer	Normal rabbit	10	0
Inulin	C6-deficient rabbit	10	0
Buffer	C6-deficient rabbit	10	0
Inulin	C6-deficient rabbit	60	22
Buffer	C6-deficient rabbit	60	33
Inulin and C6	C6-deficient rabbit	22	50
Buffer and C6	C6-deficient rabbit	22	0
Endotoxin	Normal rabbit	10	94
dH$_2$O	Normal rabbit	10	0
Endotoxin	C6-deficient rabbit	10	0
dH$_2$O	C6-deficient rabbit	10	0
Endotoxin	C6-deficient rabbit	60	16
Endotoxin	C6-deficient rabbit	60	10
Staphylococcal Protein A	Normal rabbit	12	72
Buffer	Normal rabbit	12	0
Staphylococcal Protein A	C6-deficient rabbit	12	0
Buffer	C6-deficient rabbit	12	0
Staphylococcal Protein A	C6-deficient rabbit	60	81
Buffer	C6-deficient rabbit	60	59
Heat-aggregated γ-globulin	Normal rabbit	10	73
Unheated γ-globulin	Normal rabbit	10	0
Heat-aggregated γ-globulin	C6-deficient rabbit	10	0
Unheated γ-globulin	C6-deficient rabbit	10	0
Heat-aggregated γ-globulin	C6-deficient rabbit	70	20
Unheated γ-globulin	C6-deficient rabbit	70	38
Kaolin	C6-deficient rabbit	10	>99
Buffer	C6-deficient rabbit	10	0

* Incubations were carried out at 37°C in polypropylene tubes. For additional details see Materials and Methods.

tended the incubation time sufficiently long to allow some prothrombin consumption to occur in the C6-deficient blood mixed with buffer (60 min). After this prolonged incubation, a mild anticoagulant effect of inulin became apparent and less prothrombin was consumed in the inulin-containing tubes than in

those to which buffer had been added. C6-deficient blood could be made sensitive to the clot-promoting action of inulin by the addition of small amounts (2.5 μg/ml) of isolated C6 and extending the incubation period from 10 to 22 min (Table II). The normal concentration of C6 is 25–50 μg/ml of blood.

Endotoxin and staphylococcal protein A, the two bacterial products tested, both accelerated prothrombin consumption in normal rabbit blood but had little effect on C6-deficient blood. After 10 or 12 min incubation, respectively, greater than 70% of prothrombin was consumed in normal blood mixed with these substances, whereas none was consumed in C6-deficient blood. Even after prolonged incubation (60 min) only slight acceleration of prothrombin consumption could be detected in the deficient blood.

Human γ-globulin, aggregated by heat, also accelerated prothrombin consumption in normal rabbit blood, but failed to do so in that from the C6-deficient animal. After prolonged incubation less prothrombin was consumed in C6-deficient blood mixed with heat-aggregated γ-globulin than that mixed with the unheated γ-globulin control.

These findings demonstrate that blood coagulation initiation by these complement activators requires an intact complement system. Kaolin, on the other hand, markedly accelerated clotting times and prothrombin consumption in C6-deficient blood. We have previously shown that C6-deficient blood clots much more rapidly in glass than in plastic tubes (8). Thus, these classic surface activators of Hageman factor (factor XII) can clearly initiate clotting in the absence of an intact complement system.

DISCUSSION

The classical mechanism of complement activation involves the interaction of C1 with immunoglobulin aggregates, followed by the formation of the C3 convertase ($C\overline{4,2}$) and subsequent initiation of the C3–9 sequence. Recently, an alternate pathway of complement activation has been defined which initiates the complement sequence at C3, bypassing the earlier components (10–13). A variety of plant and bacterial polysaccharides, as well as immunoglobulin aggregates, can activate this mechanism. Inulin appears to activate complement exclusively by this mechanism (13) and endotoxin largely so (1). The complement-activating properties of staphylococcal protein A depend on complex formation with immunoglobulins (14), and therefore it probably can activate both pathways.

The efficacy of inulin in promoting clotting in normal but not C6-deficient rabbit blood indicates that activation of the alternate pathway of complement activation can initiate blood coagulation. It is not yet clear if the classic mechanism of complement activation is also effective.

Our present concept of the initiation of blood coagulation by the complement-

mediated pathway is summarized in Fig. 1. Initiation of blood coagulation by the surface activators, kaolin and glass, can proceed in the absence of C6. However, the complement activators tested (endotoxin, staphylococcal protein A, immunoglobulin aggregates, and inulin) have little or no effect on clotting unless the complement system is intact. Thus, surface activation and complement activation would appear to represent two distinct mechanisms for the initiation of blood coagulation.

The precise mechanism(s) of interaction between the complement and blood coagulation systems is (are) uncertain. Antigen-antibody complexes have been reported to activate Hageman factor (15, 16) and it has been suggested that endotoxin might also do this (17, 18). Both antigen-antibody complexes and endotoxin increase platelet factor III activity (19). Whether either of these actions are complement mediated is unclear at present. However, it is apparent

Inulin
Bacterial Endotoxin
Staphylococcal Protein A
Immunoglobulin Aggregates

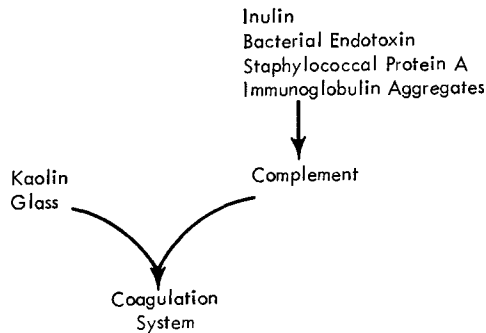Kaolin   Complement
Glass

Coagulation
System

Fig. 1. A proposed concept of the initiation of blood coagulation by the complement-mediated pathway.

that though these agents may have multiple effects on the coagulation system their predominant action is complement mediated.

The Sanarelli-Shwartzman reaction has served as the classical laboratory model for clinical intravascular coagulation of the type associated with bacterial sepsis (6). It now appears that the in vitro clot-promoting qualities of endotoxin are largely dependent on the complement-mediated pathway; it is probable that this type of intravascular coagulation is at least in part complement mediated. Several diseases characterized by the presence of circulating immunoglobulin complexes display fibrin(ogen), complement, and γ-globulin at the site of vascular or renal lesions (20-23). Complement-initiated coagulation may also play a role in the pathogenesis of these disorders. The mechanism for complement-mediated acceleration of blood coagulation and the importance of this mechanism in the pathogenesis of intravascular clotting is under investigation.

SUMMARY

A variety of complement-activating substances, including inulin, immunoglobulin aggregates, bacterial endotoxins, and staphylococcal protein A, were found to initiate blood coagulation through a complement-mediated pathway. These substances markedly accelerated blood coagulation in normal rabbit blood. That this clot-promoting activity requires an intact complement system was demonstrated by an almost total lack of effect on blood from rabbits with an inherited deficiency of the sixth component of complement (C6). Small amounts of isolated C6 conferred to C6-deficient blood the ability to respond with accelerated coagulation upon activation of the complement system. In addition, it was determined that activation of complement through the previously described C3 activator system resulted in the initiation of blood coagulation. The participation of C1, C2, and C4 was not necessary.

REFERENCES

1. Gewurz, H., H. S. Shin, and S. E. Mergenhagen. 1968. Interactions of the complement system with endotoxin lipopolysaccharides: consumption of each of the six terminal complement components. *J. Exp. Med.* **128**:1049.
2. Ishizaka, T., K. Ishizaka, S. Salmon, and H. Fudenberg. 1967. Biologic activities of aggregated γ-globulin. VIII. Aggregated immunoglobulins of different classes. *J. Immunol.* **99**:82.
3. McKay, D. G., S. S. Shapio, and J. N. Shanberg. 1958. Alterations in the blood coagulation system induced by bacterial endotoxins. II. In vitro. *J. Exp. Med.* **107**:369.
4. Robbins, J., and C. A. Stetson. 1959. An effect of antigen-antibody interaction on blood coagulation. *J. Exp. Med.* **109**:1.
5. Becker, E. L. 1969. The relations of complement to other systems. *Proc. Roy. Soc. Ser. B. Biol. Sci.* **173**:383.
6. Hjort, P. F., and S. I. Rapaport. 1965. The Shwartzman reaction: pathogenetic mechanisms and clinical manifestations. *Annu. Rev. Med.* **16**:135.
7. Ratnoff, O. D. 1969. Some relationships among hemostasis, fibrinolytic phenomena, immunity, and the inflammatory response. *Advan. Immunol.* **10**:145.
8. Zimmerman, T. S., C. M. Arroyave, and H. J. Müller-Eberhard. 1971. A blood coagulation abnormality in rabbits deficient in the sixth component of complement (C6) and its correction by purified C6. *J. Exp. Med.* **134**:1591.
9. Arroyave, C. M., and H. J. Müller-Eberhard. 1971. Isolation of the sixth component of complement from human serum. *Immunochemistry.* In press.
10. Oliveira, B., A. G. Osler, R. P. Siraganian, and A. L. Sandberg. 1970. The biologic activities of guinea pig antibodies. I. Separation of γ1 and γ2 immunoglobulins and their participation in allergic reactions of the immediate type. *J. Immunol.* **104**:320.
11. Sandberg, A. L., A. G. Osler, H. S. Shin, and B. Oliveira. 1970. The biologic activities of guinea pig antibodies. II. Modes of complement interaction with γ1 and γ2 immunoglobulins. *J. Immunol.* **104**:329.

12. Ellman, L., I. Green, F. Judge, and M. M. Frank. 1971. In vivo studies in C4-deficient guinea pigs. *J. Exp. Med.* **134**:162.

13. Götze, O., and H. J. Müller-Eberhard. 1971. The C3-activator system: an alternate pathway of complement activation. *J. Exp. Med.* **134** (3, Pt. 2):90.

14. Sjöquist, J., and G. Stålenheim. 1969. Protein A from *Staphylococcus aureus*. IX. Complement-fixing activity of protein A-IgG complexes. *J. Immunol.* **103**:467.

15. Movat, H. Z., and N. L. DiLorenza. 1968. Activation of the plasma kinin system by antigen-antibody aggregates. I. Generation of permeability factor in guinea pig serum. *Lab. Invest.* **19**:187.

16. Kaplan, A. P., I. Gigli, and K. F. Austen. 1971. Immunologic activation of Hageman factor and its relationship to fibrinolysis, bradykinin generation and complement. *J. Clin. Invest.* **50**:51a. (Abstr.)

17. McKay, D. G., G. Muller-Berghaus, and V. Cruse. 1969. Activation of Hageman factor by ellagic acid and the generalized Shwartzman reaction. *Amer. J. Pathol.* **54**:393.

18. Pettinger, W. A., and R. Young. 1970. Endotoxin induced kinin (bradykinin) formation: activation of Hageman factor and plasma kallikrein in human blood. *Life Sci.* **9**:313.

19. Horowitz, H. I., R. M. DesPrez, and E. W. Hook. 1962. Activation of platelet factor III activity in vivo and in vitro. *J. Exp. Med.* **116**:619.

20. Cochrane, C. G., and F. J. Dixon. 1968. Cell and tissue damage through antigen-antibody complexes. *In* Textbook of Immunopathology. P. A. Miescher and H. J. Müller-Eberhard, editors. Grune & Stratton Inc., New York. **1**:94.

21. Paronetto, F., and D. Koffler. 1964. Immunofluorescent localization of immunoglobulins, complement, and fibrinogen in human diseases. I. Systemic lupus erythrematosus. *J. Clin. Invest.* **44**:1657.

22. Paronetto, F. 1969. Systemic nonsuppurative necrotizing angiitis. *In* Textbook of Immunopathology. P. A. Miescher and H. J. Müller-Eberhard, editors. Grune & Stratton Inc., New York. **2**:722.

23. McCluskey, R. T., P. Vassali, G. Gallo, and D. S. Baldwin. 1966. An immunofluorescent study of pathogenic mechanisms in glomerular diseases. *N. Engl. J. Med.* **274**:695.