

A BLOOD COAGULATION ABNORMALITY IN RABBITS DEFICIENT
IN THE SIXTH COMPONENT OF COMPLEMENT (C6)
AND ITS CORRECTION BY PURIFIED C6*

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A strain of rabbits with an inherited complement defect (1) has been shown to be deficient in the sixth component of complement (C6) hemolytic activity (2, 3). No protein immunochemically related to C6 is present in plasma from these animals and they are able to produce antibody to rabbit C6 (2, 4).

In this communication we report investigations of a coagulation abnormality we have found in blood from these animals. Our results provide direct evidence for an involvement of C6 in normal blood coagulation.

Materials and Methods

Venous Blood.—Venous blood from normal New Zealand white rabbits and rabbits deficient in C6 (1) (purchased from Rancho de Conejo, Vista, Calif.) was withdrawn from the hind leg vein with a 19 gauge small vein infusion set (McGaw Laboratories Inc., Glendale, Calif.) and disposable polypropylene syringes (Sherwood Medical Industries Inc., Deland, Fla.). Blood for measurement of whole blood clotting time and prothrombin consumption was immediately placed in the appropriate incubation tubes (see below). Blood drawn from ear arteries had consistently shorter clotting times than leg vein blood, suggesting contamination with tissue juices. No more than 12 ml was withdrawn at one time as the prolonged venesection required for obtaining larger quantities of blood was associated with a shortening of the clotting time.

Blood to provide plasma for coagulation studies was mixed with one-fiftieth volume of 0.5 M sodium citrate buffer (pH 5.0) in 12 × 75-mm polypropylene tubes (Falcon Plastics, Oxnard, Calif.). Platelet-poor plasma was separated by centrifugation at 3500 g for 15 min in an International refrigerated centrifuge, PR-2 (International Equipment Company, Boston, Mass.). Platelet-rich plasma was separated from whole blood by centrifugation at a force of 850 g for 10 min.

Clotting Time.—Clotting time of 1 ml aliquots of whole blood was determined in 10 × 75-mm disposable glass culture tubes (Corning Glass Works, Corning, N.Y.) and 12 × 75-mm polypropylene tubes (Falcon Plastics) at 25°C (5). A serial, three-tube technique was used

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with observation for clotting every minute in glass tubes and every 10 min in polypropylene tubes. Tubes were tapped against the side of the water bath to determine when a solid clot filled the entire volume. Formation of this solid clot was taken as the end point.

The effect of highly purified C6 on whole blood clotting time in polypropylene tubes was determined by adding C6 or buffer as a control to each of three tubes before adding the blood. The tubes were then tapped gently to achieve mixing and observed for clotting.

Recalcified clotting time of 0.2 ml plasma aliquots was determined in duplicate 10 × 70-mm disposable glass tubes. Plasma was kept at 4°C until addition of 0.2 ml of 0.025 M CaCl₂, also kept at 4°C. The tubes were then transferred immediately to a 25°C water bath and observed for clotting every 60 sec.¹

Prothrombin (Factor II) Consumption.—Protrombin (factor II) consumption was determined in triplicate 1 ml aliquots of whole blood and results are expressed as the mean. The blood was incubated in three 12 × 75-mm polypropylene tubes for 60 min at 37°C, then placed in an ice water bath and 0.04 ml of 0.5 M sodium citrate added to each tube. The clots were broken up with birch applicator sticks and the tubes incubated at 37°C for 30 min to inactivate thrombin. After centrifugation at 3500 g, the supernatant serum was assayed for prothrombin (see below). 1 ml of blood, obtained at the same time as the previous samples, was also added to 0.04 ml of 0.5 M sodium citrate in a 12 × 75 mm polypropylene tube and carried through the same incubations. The prothrombin activity in the plasma from this blood was assigned the value of 100% and appropriate dilutions were used to construct a calibration curve. Prothrombin consumption was calculated by subtracting the per cent prothrombin detectable on assay from 100.

The effect of highly purified C6 on prothrombin consumption in whole blood was measured by adding either C6 or buffer to duplicate tubes before the addition of blood. Mixing was achieved by gentle tapping. This agitation sometimes accelerated prothrombin consumption in tubes containing buffer only. Incubation times were therefore shortened to approximately 40 min so that less than 5% prothrombin was consumed in tubes containing buffer only.

Other Coagulation Assays.—The partial thromboplastin time without kaolin, prothrombin time, and assays for stable factor (factor VII), prothrombin (factor II), and Stuart factor (factor X) were performed as described in a standard text (6) using artificial substrates for factor II and factor X, as indicated in the text and using canine plasma congenitally deficient in factor VII for assay of that factor. Labile factor (factor V) was assayed in the same manner, except that aged, oxalated human plasma (7) was used as a substrate. The assays of Hageman factor (factor XII), plasma thromboplastin antecedent (PTA) (factor XI), Christmas factor (factor IX), and antihemophilic factor (factor VIII) were performed as described (8) using as substrates human plasma congenitally deficient in the factor being assayed. Thrombin time (9), Russell's viper venom time of unabsorbed platelet-poor plasma (10), thromboplastin generation test (11, 12), kaolin-activated Russell's viper venom time of platelet-rich plasma (13), clot retraction of blood clotted with tissue thromboplastin (14), platelet count (15), and platelet aggregation (16) also were performed by techniques described by others. Bleeding time was measured after incision of a toe pad with a No. 11 scalpel blade (Bard-Parker, Rutherford, N.J.). Blood was removed from the wound every 20 sec with filter paper, taking care not to touch the wound itself.¹

Isolated C6.—The method of isolation of C6 has been described in detail elsewhere (17). 1 ml aliquots of isolated C6 were dialyzed against 1000 ml of tris(hydroxymethyl)amino-methane (Tris) saline buffer (0.05 M Tris, 0.1 M sodium chloride, pH 7.0) or barbital-buffered saline (0.05 M sodium barbital, 0.1 M sodium chloride, pH 7.5) for 24 hr before use and the dialysis buffer was used as control buffer for these studies.

Molecular Sieve Chromatography.—850 μg of highly purified C6 or 4000 μg of partially

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purified C6 were applied to a 5×100 cm Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) column equilibrated with barbital-buffered saline and the effluent was collected in 2-ml fractions at a flow rate of 30 ml/hr.

C6 Hemolytic Activity.—C6 hemolytic activity was measured in a 0.5 ml reaction mixture containing 100 μ l sample, C6-deficient rabbit serum in a final dilution of 1:20 and 5×10^7 sensitized sheep erythrocytes, or with sensitized cells and purified complement components (17). 0.15 M barbital buffer, pH 7.5, was used which contained 0.00015 M CaCl_2 and 0.000059 M MgCl_2 . The mixture was incubated at 37°C for 30 min and then brought to 2 ml with cold (4°C) saline and centrifuged. The degree of hemolysis was determined by measuring free hemoglobin in the supernatant at 541 μ on a Coleman Jr. II spectrophotometer, Model 635 (Coleman Instruments, Maywood, Ill.).

Consumption of Complement Component Activities.—Consumption of complement component activities during blood coagulation was determined on human venous blood drawn from the antecubital vein with a 19 gauge small vein infusion set and disposable polypropylene syringes. The blood was treated as for the determination of prothrombin consumption with the exception that 10 \times 75-mm glass tubes were used for incubation. Except where indicated, the blood was allowed to clot for 2 hr at 37°C. The complement activities in serum were then compared with the activities in citrated plasma which had been allowed to incubate for 2 hr at 37°C before separation from the formed blood elements.

Assays of Complement Components.—Assays of complement components C2, C8, C9 (18), and C7² have been described elsewhere.

Protein.—Protein was measured by the method of Lowry et al. (19) using purified C3 as a standard.

Polyacrylamide-Gel Electrophoresis.—Polyacrylamide-gel electrophoresis was carried out by the method of Davis (20) employing a polyacrylamide concentration of 6% in the separation gel. Buffalo black was used as the stain.

RESULTS

Blood Coagulation Studies in C6-Deficient and Normal Rabbits.—Clotting time of whole blood in glass and plastic tubes was consistently prolonged in C6-deficient rabbits and prothrombin consumption markedly decreased (Table I). When normal and C6-deficient rabbit blood were mixed in equal quantities before incubation, partial correction of the prothrombin consumption abnormality was achieved. Clotting time of blood from a C6-deficient rabbit, blood from a normal rabbit, and a mixture of equal parts of C6-deficient and normal blood were respectively >230, 80, and 110 min. Prothrombin consumption was 0, 60, and 41 %.

No other coagulation abnormalities were demonstrated. In a typical C6-deficient rabbit the prothrombin time was 12.4 sec with a control of 12.2 sec. The partial thromboplastin time was 58 sec with a control of 62 sec. The recalcified clotting time was 4 min with a control of 3.5 min. The Russell's viper venom time on platelet-poor plasma was 12.6 sec with a control of 12.2 sec. Thrombin time was 26 sec with a control of 30 sec. Specific coagulation factor activity assays were all normal. The factor II (prothrombin) activity was 100 %

² Arroyave, C. M., and H. J. Müller-Eberhard. Manuscript in preparation.

the factor V (proaccelerin), 100%; the factor VII (stable factor), 70%; the factor VIII (antihemophilic factor), 80%; the factor IX (Christmas factor), >100%; the factor X (Stuart factor), 100%; the factor XI (PTA), >100%; and factor XII (Hageman factor), >100% of a normal rabbit.

Platelet factor III activity as measured in the thromboplastin generation test and kaolin-activated Russell's viper venom time was also normal. In the 6 min thromboplastin generation test using autologous platelets, the substrate clotting time was 20 sec with a control of 19 sec. The kaolin-activated Russell's viper venom time was 18 sec with a control of 20 sec in platelet-rich plasma. Other normal platelet parameters include a platelet count of 485,000 with a control of 525,000 and normal platelet aggregation with adenosine 5'-diphosphate (Sigma Chemical Co., St. Louis, Mo.) and collagen (Sigma). Clot retraction of blood clotted with tissue thromboplastin was normal with 78% of serum expressed

TABLE I
*Abnormal Clotting Time and Prothrombin Consumption in C6-Deficient rabbits**

Clotting time				Prothrombin consumption	
C6 deficient		Normal		C6 Deficient	Normal
Glass	Plastic	Glass	Plastic		
	<i>min</i>				<i>%</i>
39	>400	23	90	0	78.5
34	170	20	90	4	75.5
38	>340	24	90	0	76
41	160	24	70	0	75
40	140	31	70	0	68
				10	60

* Each value represents a determination on an individual animal.

from the clot as compared to 80% in the control. Bleeding time was 180 sec with a control of 160 sec.

The Effect of Highly Purified C6 on Blood Coagulation in C6-Deficient Rabbits.—Preparations of highly purified human or rabbit C6 were analyzed for homogeneity by polyacrylamide-gel electrophoresis. No contaminants could be detected in the 50 to 100- μ g samples applied to the gels (Fig. 1). There was no detectable clotting factor activity in a typical preparation containing 450 μ g of protein/ml which was assayed undiluted (Table II).

Addition of highly purified C6 to blood of C6-deficient rabbits markedly shortened the clotting time and accelerated the prothrombin consumption. Addition of 30 μ g of C6 in 0.25 ml of buffer shortened the plastic tube clotting time to 100 min from 200 min (when buffer alone was added). Prothrombin consumption was increased to 80% when 45 μ g of C6 in 0.1 ml of buffer was added. No prothrombin was consumed when only buffer was added. The effect

of C6 on prothrombin consumption is a function of the amount of C6 added (Fig. 2). The clot-promoting activity was detectable even in amounts less than the quantities present in normal blood (25–50 $\mu\text{g}/\text{ml}$ of blood).³

An effect of highly purified C6 could also be demonstrated on normal rabbit blood if incubation times were shortened. When 1 ml of normal blood was incubated with 0.05 ml of buffer for 15 min, 30% of prothrombin was consumed as opposed to 88% when C6 (450 $\mu\text{g}/\text{ml}$ of buffer) was added.

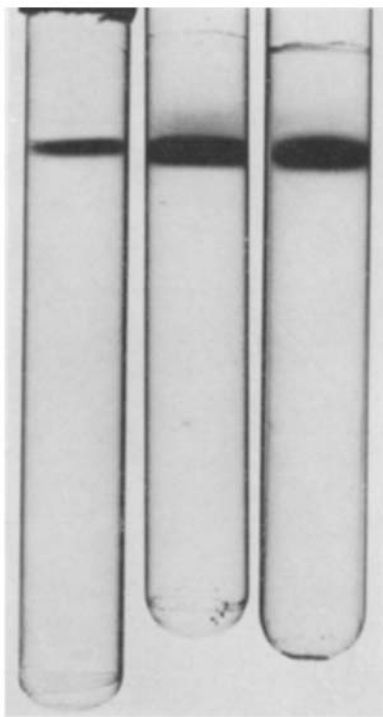


FIG. 1. Polyacrylamide-gel electrophoresis of C6 preparations. The first preparation (left) was obtained from rabbit serum, the second two from human serum.

The C6 hemolytic and clot-promoting activities remained together during the purification process. Filtration of a partially purified preparation through a Sephadex G-200 column resulted in the separation of most of the protein from the hemolytic and clot-promoting activities, but not in separation of these activities from each other (Fig. 3). When a highly purified preparation with only one protein band on polyacrylamide-gel electrophoresis was similarly subjected to molecular sieve chromatography, an excellent correlation was observed be-

³ Arroyave, C. M. Unpublished data.

tween the distribution of protein, C6 hemolytic activity, and clot-promoting activity (Fig. 4).

Consumption of Complement Component Hemolytic Activities during Blood Coagulation.—Both C6 and C7 hemolytic activities were partially consumed during the process of blood coagulation. C6 activity diminished 13.3–14.5%

TABLE II
*Lack of Classical Clotting Factor Activity in a Highly Purified Preparation of C6**

Factor	Units of activity†
II	<0.01
V	<0.01
VII	<0.01
VIII	<0.01
IX	<0.01
X	<0.01
XI	<0.01
XII	<0.01

* The protein concentration was 450 $\mu\text{g}/\text{ml}$.

† 1 unit of activity is defined as the activity in 1 ml normal rabbit plasma.

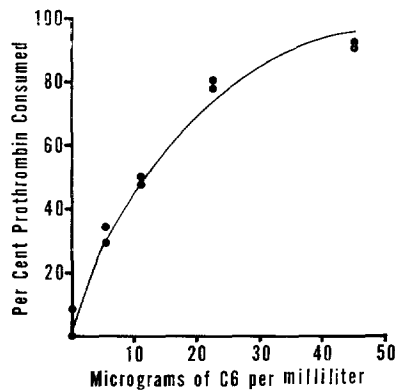


FIG. 2. The relationship of prothrombin consumption in C6-deficient plasma to the quantity of isolated C6 added.

and C7 decreased 7–10% during 10 separate experiments. A similarity between the time course of consumption of C6 hemolytic activity and prothrombin was observed (Fig. 5). No consumption of C2, C8, or C9 was demonstrated.

DISCUSSION

The demonstration of prolonged clotting times and retarded prothrombin consumption in rabbits deficient in the sixth component of complement and the

correction of these abnormalities with highly purified C6 suggests that this protein is a normal participant in the blood coagulation mechanism. The partial consumption of C6 hemolytic activity during blood coagulation with a time course parallel to that of prothrombin consumption provides further evidence for this concept.

It is possible, but improbable, that, in addition to the C6 deficiency, another

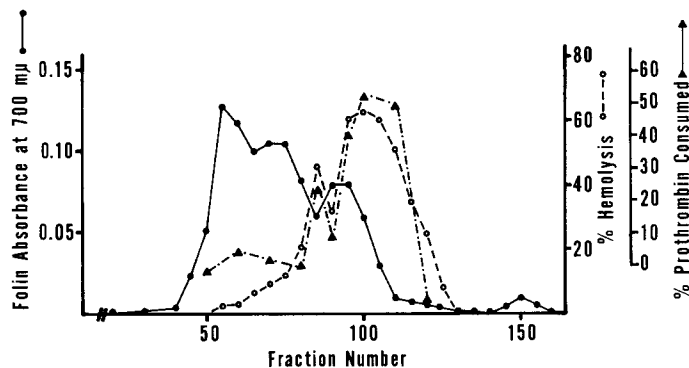


FIG. 3. Distribution of protein, C6 hemolytic activity, and clot-promoting activity upon molecular sieve chromatography of partially purified C6 on a Sephadex G-200 column.

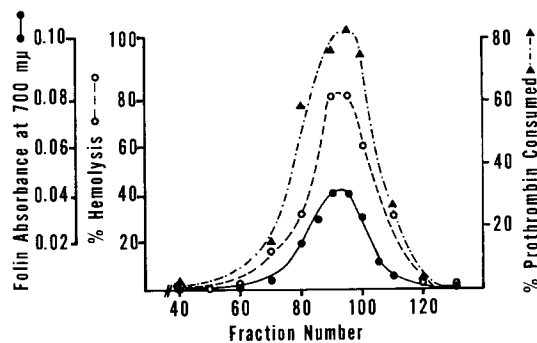


FIG. 4. Distribution of protein, C6 hemolytic activity, and clot-promoting activity upon molecular sieve chromatography of highly purified C6 on a Sephadex G-200 column.

genetic abnormality is present in these animals accounting for the coagulation disorder. Such an explanation implies that the second gene product is indistinguishable from C6 by present physicochemical techniques. It seems unlikely that the clot-promoting effect of the C6 preparations is due to the presence of a contaminating protein. The C6 hemolytic and clot-promoting activities remained associated during the purification of the protein, and no classical clotting factor activity could be detected in the highly purified preparations. The clot-pro-

moting activity of highly purified C6 strongly implies that the absence of C6 protein in the deficient rabbits is responsible for their coagulation abnormality.

A possible role of complement in blood coagulation has been previously suggested by the demonstration of shortening of whole rabbit blood clotting time with antigen-antibody complexes (21). The participation of complement (C3 and C4) in the lysis of dilute blood clots has been demonstrated previously (22).

Our findings show that C6 may be involved in blood coagulation even without the participation of antigen-antibody reactions, and thus indicate a role in normal blood clotting. The partial consumption of C7, as well as C6, during blood coagulation suggests that both components may be involved. The exact mechanism of complement participation in coagulation and possible involvements of other complement components is under study.

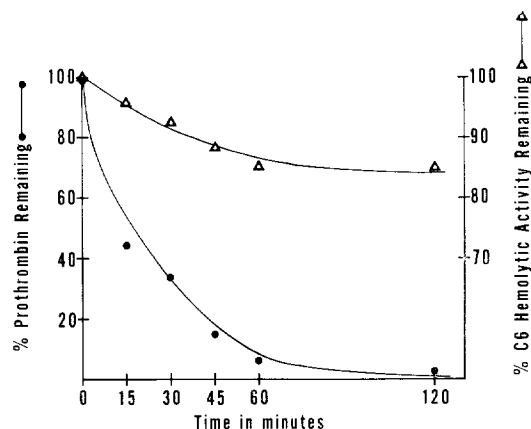


FIG. 5. Time course of consumption of C6 hemolytic activity and prothrombin during the clotting of human blood.

Complement deficiencies may be in part responsible for coagulation disorders which have not yet been adequately explained by abnormalities of the classically described factors. In addition, experiments reported in the accompanying communication indicate that activation of complement may initiate blood coagulation, thus suggesting an important role for complement in intravascular coagulation syndromes.

SUMMARY

Evidence for the involvement of the sixth component of complement (C6) in normal blood coagulation is provided by the description of a coagulation abnormality in rabbits with a genetic C6 deficiency and by its correction with highly purified preparations of C6. Whole blood clotting time in glass or plastic was prolonged and prothrombin consumption was decreased in blood from the

deficient animals. Other parameters of blood coagulation were normal, including prothrombin time, partial thromboplastin time, specific clotting factor activities, platelet factor III function, platelet count, and bleeding time. Clotting time and prothrombin consumption became normal when physiologic amounts of highly purified C6 were added to the deficient blood. Partial consumption of C6 hemolytic activity, with a time course similar to the consumption of prothrombin, was demonstrated during the clotting of normal human blood.

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REFERENCES

1. Rother, U., and K. Rother. 1961. Über einen angeborenen Komplementdefekt bei Kaninchen. *Z. Immunitätsforsch Allergie Klin. Immunol.* **121**:224.
2. Rother, K., U. Rother, H. J. Müller-Eberhard, and U. R. Nilsson. 1966. Deficiency of the sixth component of complement in rabbits with an inherited complement defect. *J. Exp. Med.* **124**:773.
3. Nelson, R. A., Jr., and C. E. Biro. 1968. Complement components of a hemolytically deficient strain of rabbits. *Immunology.* **14**:527.
4. Lachmann, P. J. 1970. C6 deficiency in rabbits. *In* Protides of the Biological Fluids; XVII Colloquium. H. Peeters, editor. Pergamon Press Inc., New York. 301.
5. Ratnoff, O. D. 1960. Bleeding syndromes. Charles C Thomas Publisher, Springfield, Ill. 19.
6. Tocantins, L. M., and L. A. Kazal. 1965. Blood Coagulation in Hemorrhage and Thrombosis, Methods of Study. Grune & Stratton Inc., New York. 103, 148, 144, 213, 129.
7. Stormorken, H. 1957. The preparation of proaccelerin deficient (parahemophilia) plasma for the assay of proaccelerin. *Scand. J. Clin. Lab. Invest.* **9**:273.
8. Ratnoff, O. D., and E. W. Davie. 1962. The purification of activated Hageman factor (activated factor XII). *Biochemistry.* **1**:967.
9. Ratnoff, O. D. 1954. An accelerating property of plasma for the coagulation of fibrinogen by thrombin. *J. Clin. Invest.* **33**:1175.
10. Hjort, P., S. I. Rapaport, and P. Owen. 1955. A simple, specific one stage prothrombin assay using Russell's viper venom in cephalin suspension. *J. Lab. Clin. Med.* **46**:89.
11. Bowie, E. J. W., J. H. Thompson, and C. A. Owen, Jr. 1965. Standardization of the platelets in the thromboplastin generation test. *Amer. J. Clin. Pathol.* **44**:673.
12. Duckert, F., P. Flückiger, H. Isenschmid, M. Matter, J. Vogel-Meng, and F. Koller. 1954. A modification of the thromboplastin generation test. *Acta Haematol.* **12**:197.
13. Spaet, T. H., and J. Cintron. 1965. Studies on platelet factor-3 availability. *Brit. J. Haematol.* **11**:269.

14. Jackson, D. P., R. C. Hartman, and C. L. Conley. 1953. Clot retraction as a measure of platelet function. *Bull. Johns Hopkins Hosp.* **93**:370.
15. Tocantins, L. M., and L. A. Kazal. 1965. Blood Coagulation in Hemorrhage and Thrombosis, Methods of Study. Grune & Stratton Inc., New York. 52.
16. Mustard, J. F., B. Hegardt, H. C. Rowsell, and R. L. MacMillan. 1964. Effect of adenosine nucleotides on platelet aggregation and clotting time. *J. Lab. Clin. Med.* **64**:548.
17. Arroyave, C. M., and H. J. Müller-Eberhard. 1971. Isolation of the sixth component of complement from human serum. *Immunochemistry*. In press.
18. Müller-Eberhard, H. J. 1971. Methods of isolation and assay of human complement components. In *Methods in Immunology and Immunochemistry*. C. A. Williams and M. W. Chase, editors. Academic Press Inc., New York. **4**. In press.
19. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin reagent. *J. Biol. Chem.* **193**:265.
20. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**:404.
21. Robbins, J., and C. A. Stetson. 1959. An effect of antigen-antibody interaction on blood coagulation. *J. Exp. Med.* **109**:1.
22. Taylor, F. B., Jr., and H. J. Müller-Eberhard. 1967. Factors influencing lysis of whole blood clots. *Nature (London)*. **216**:1023.