Hereditary Deficiency of the Sixth Component of Complement in Man

II. STUDIES OF HEMOSTASIS

R. S. HEUSINKVELD, J. P. LEDDY, M. R. KLEMPERER, and R. T. BRECKENRIDGE

From the Departments of Medicine, Pediatrics and Microbiology, Strong Memorial Hospital and Rochester General Hospital, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

ABSTRACT Prompted by previous observations of defective blood clotting in rabbits deficient in the sixth component of complement (C6), an evaluation was made of the hemostatic functions of the homozygous proband of a newly recognized human kindred with hereditary C6 deficiency. This human subject, who had no clinical evidence of a bleeding disorder, exhibited a total lack of C6 by functional and immunoprecipitin assays of serum or plasma.

Standard tests of hemostatic function were normal; however, when the whole blood clotting time was measured at 25°C in plastic tubes, it was at the upper range of our normal values. In confirmation of this observation, prothrombin consumption, when performed at 37°C in plastic tubes, was at the lower range of normal. Inulin and endotoxin, in concentrations shown to cause activation of human complement, had little or no effect on clotting times or prothrombin consumption of normal or C6-deficient human blood.

These observations indicate that absence of C6 does not have a significant effect on hemostatic function in man. In the light of other investigations, the observed differences in clotting function between C6-deficient human blood and C6-deficient rabbit blood could be due to species differences governing the susceptibility of platelets to complement activation.

This work was presented in part at the annual meeting of the American Society for Clinical Investigation, Atlantic City, N. J., 29 April 1973 (1973. *J. Clin. Invest.* **52**: 50 *a*. [Abstr.]) and at the 5th International Complement Workshop, Coronado, Calif., 25 February 1973.

Dr. Heusinkveld's current address is the Department of Radiation-Oncology, University of Arizona Medical Center, Tucson, Ariz. 85721.

Reprint requests should be directed to Dr. Leddy at the University of Rochester Medical Center, Rochester, N. Y. 14642.

Received for publication 28 June 1973 and in revised form 7 September 1973.

INTRODUCTION

A variety of clinical and laboratory observations indicate that the hemostatic and immune systems may interact at several levels (1–3). Indeed, a direct role for components of the complement (C)[1] system in blood coagulation has been suggested by the finding that complement-activating substances such as endotoxin and antigen-antibody complexes accelerate clotting (4–6).

Recently, more specific implication of late-acting components of the complement sequence in clotting has resulted from the discovery that the rate of blood coagulation is retarded in rabbits genetically deficient in C6 (7, 8). The clotting time and prothrombin consumption of whole blood from C6-deficient (C6D) rabbits were found to be abnormal in both glass and plastic tubes. Moreover, complement-activating substances which accelerate coagulation and prothrombin consumption of normal rabbit blood did not appreciably shorten the clotting time or increase the rate of prothrombin consumption of C6D rabbit blood. The coagulation defects of C6D rabbit blood were corrected by addition of purified C6. Because the usual assays of platelet function and clotting factor activity of C6D rabbit blood were normal, it was suggested that complement per se

[1] *Abbreviations used in this paper:* C, complement; C6D, C6-deficient; PRP, platelet-rich plasma; VBS, Veronal-buffered saline.

may play a role in normal coagulation, and that some unexplained hemorrhagic diatheses in man may be related to complement deficiencies (7).

The discovery of a human subject whose serum and plasma lacked detectable C6 (9) has provided a unique opportunity to assess the role of complement in human blood coagulation. There was no clinical evidence that this C6D proband had a hemorrhagic diathesis.

METHODS

Blood. Blood for clotting and platelet studies was obtained in the following manner: A no. 20 disposable needle connected to a 20-inch Venotube (Abbott Laboratories, North Chicago, Ill.) was inserted into a large antecubital vein. Immediately after venipuncture, the tourniquet, loosely applied to the upper arm, was removed. 30 s after removal of the tourniquet, blood was allowed to fill the venotube which was rinsed by removal of 5 ml of blood into a syringe. A second disposable plastic syringe (Tomac, American Hospital Supply, Evanston, Ill.) was then connected to the Venotube and a maximum of 25 ml of blood were withdrawn slowly for clotting studies. Only blood from atraumatic venipunctures was used since even the slightest difficulty in entering a vein was found to markedly shorten the plastic clotting times.

Plasmas. Plasmas from normals and from patients with various coagulation abnormalities were collected in uncoated plastic tubes, prepared in a manner published previously (10), and stored at −70°C until used.

Clotting times. Clotting times were determined at both 25°C and 37°C as described by Zimmerman, Arroyave, and Müller-Eberhard (7) using disposable 10 × 75 mm glass round bottom tubes (Rochester Scientific Co., Rochester, N. Y.), 12 × 75 mm polypropylene tubes, and 12 × 75 mm polystyrene tubes (both from Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The end point was defined as the formation of a solid clot, filling the entire volume of blood, which could not be broken up by gentle tapping of an inverted tube.

Prothrombin consumption. Prothrombin consumption in normal blood samples and in C6D blood was determined in glass, polypropylene, and polystyrene tubes at 37°C according to a modification of the method of Quick and Favre-Gilly (11). Three 2.25-ml aliquots of blood were incubated at 37°C for varying intervals. Several incubation periods were chosen in an attempt to identify differences in the rate of prothrombin consumption between normal and C6D blood. A fourth 2.25-ml aliquot of blood was anticoagulated with 0.25 ml citrate anticoagulant (0.13 M Na citrate-citric acid, pH 5.0) and incubated under the same conditions. At the end of the incubation period, 0.25 ml citrate anticoagulant was added to each of the three tubes which originally contained no anticoagulant. The four tubes were then placed in an ice water bath and the clots, if any, were thoroughly homogenized. The four tubes were then reincubated at 37°C for 30 min. Supernatant plasmas were obtained by centrifugation at 4,000 *g* for 10 min at 4°C and the prothrombin content determined (12). The prothrombin content of the experimental tubes was expressed in terms of the anticoagulated aliquot which was arbitrarily assigned a value of 100%.

The thromboplastin and activated cephaloplastin used were purchased from the Dade Div., American Hospital Supply Corp., Miami, Fla.

Platelet studies. Platelet aggregation induced by final concentrations of 0.1 μg/ml ADP, 0.04 μg/ml collagen, and 0.01 M epinephrine was determined with an aggregometer (model S201, Chrono-Log Corp., Broomall, Pa.) as previously described (13). The results are expressed in terms of the time required to reach maximum aggregation. Several hundred normal individuals have been examined by this technique and the normal range is listed in Table I.

Clot retraction was measured by a previously described method (14). The method of Spaet and Cintron utilizing Russell's viper venom was used for the measurement of platelet Factor III release (15). The incubation of platelet-rich plasma (PRP) and kaolin was continued for 30 min at 37°C and then a venom clotting time determined. This 30-min clotting time was then expressed as a percent of the clotting time determined after freeze thawing of the fresh PRP which was arbitrarily designated as 100% release. 20 normal individuals were examined in an identical fashion and the normal range expressed in Table I.

Specific assays. Fibrinogen (Factor I) was determined by the method of Ingram (16). Prothrombin (Factor II) was measured by the use of an artificial substrate (12). Fibrin-stabilizing factor (FSF, Factor XIII) was estimated by the solubility of the clot in 5 M urea (17). Proaccelerin (Factor V), pro-SPCA (Factor VII), antihemophilic factor (Factor VIII), Christmas factor (Factor IX), Stuart factor (Factor X), plasma thromboplastin antecedent (PTA, Factor XI), and Hageman factor (Factor XII) were determined by previously published methods using plasma from patients with congenital factor deficiencies as substrates (10, 18). The activated partial thromboplastin times and prothrombin times were determined according to standard techniques (19, 20).

The effect of inulin and endotoxin on the clotting time and rate of prothrombin consumption of normal and C6D human blood were assayed exactly as described by Zimmerman and Müller-Eberhard (8). Shigella endotoxin was the gift of Dr. Gabriel Michael, University of Cincinnati School of Medicine, Cincinnati, Ohio. Inulin (white purified) was purchased from Fisher Scientific Co., Fair Lawn, N. J. Endotoxin was dissolved in Veronal-buffered saline, pH 7.5, and inulin was dissolved in distilled water (8). The concentrations of endotoxin and inulin given in the legends of Figs. 3–5 are final concentrations.

These endotoxin and inulin preparations were shown, at the concentrations used in the coagulation tests, to activate complement by the following criteria: (*a*) immunoelectrophoretic conversion of C3 proactivator (glycine-rich β-globulin, properdin factor B) in human serum,[2] (*b*) generation of chemotactic factors from fresh serum,[3] and (*c*) depletion of whole complement titers in human serum.

Complement assays. CH_{50} titers were performed on normal donors as described previously (21). As an estimate of C6 in such sera, "$C6H_{50}$ titers" were determined as follows: to 0.2 ml sensitized sheep red blood cells (5×10^7 cells/ml) were added 0.4 ml Veronal-buffered saline (VBS), 0.4 ml 1/50 dilution (in VBS) of C6D human serum (patient D. B.) and 0.4 ml of diluted test serum (1/10,000, 1/20,000, 1/30,000, and 1/40,000 in VBS); the remainder of the procedure was the same as with CH_{50} titers. Straight line plots were obtained when percent lysis (as probits) was related to test serum dilutions (log scale). The serum dilution producing 50% lysis was read graphically and con-

[2] Kindly determined by Dr. Michael Frank, NIH.

[3] Assayed by Dr. John Baum, University of Rochester.

TABLE I

Survey of Hemostatic Functions of C6-Deficient Human

	C6-deficient subject	Normal range
Coagulation studies		
Activated partial thromboplastin time	40.5 s	32–50 s
Prothrombin time	12.9 s	12–14 s
Whole blood clotting time		
Glass, 25°C (10 × 75 mm tubes)	24 min	18–33 min
Polystyrene, 37°C	64 min	23–70 min
Prothrombin consumption		
Glass, 37°C, 1 h	>99%	>75%
Platelet studies		
Platelet count	200,000/mm³	150–400,000/mm³
Platelet aggregation		
Collagen	106 s	60–120 s
ADP	11.0 s	10–20 s
Epinephrine	12.5 s	10–20 s
Clot retraction, 37°C	63%	40–70%
Platelet Factor III release		
Platelet-rich plasma	70%	50–97%
Whole blood	72%	
Assays for individual clotting factors		
I–XIII	Normal	

verted to units per milliliter as described (21). The normal range was established by preliminary study of 20 normal donors.

RESULTS

By standard methods an extensive evaluation of the C6D patient's hemostatic system was entirely within normal limits (Table I). Further attempts to identify a hemostatic defect in the C6D human were patterned after methods which had demonstrated abnormalities in C6D rabbits (7, 8). Fig. 1 illustrates that in polypropylene tubes and, in a smaller series, in polystyrene tubes clotting times of the C6D human blood at 25°C fell within the upper range of normal subjects tested.

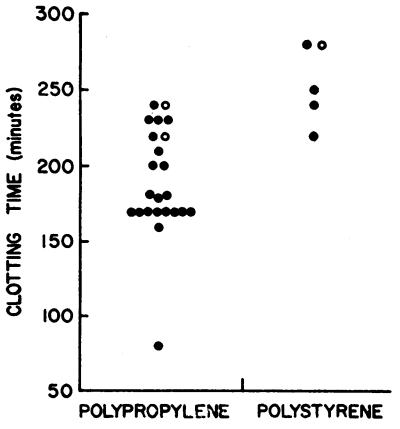

FIGURE 1 Whole blood clotting times in polypropylene and polystyrene plastic tubes at 25°C. ●, normals; ○, C6D.

Similarly, when prothrombin consumption was tested in plastic tubes at two time intervals (Fig. 2), the rate of consumption in C6D human blood was within normal range, although consistently at the lower limit of normal.

The sera of the six normal donors with the lowest rates of prothrombin consumption or longest plastic clotting times were shown to have normal whole hemolytic complement (CH_{50}) titers and $C6H_{50}$ titers. The latter assay is based on the capacity of a highly diluted donor serum to restore the hemolytic activity of the C6D human serum (see Methods). Thus, the normal donors whose coagulation functions overlapped those of the C6D patient did not appear to be deficient in C6.

In the studies of Zimmerman and Müller-Eberhard (8), a particularly striking defect of C6D rabbit blood was its failure to show the marked acceleration of clotting and prothrombin consumption observed with normal rabbit blood in the presence of inulin, endotoxin, or other complement-activating substances. As shown in Fig. 3, the clotting time of normal human blood in polypropylene tubes was not significantly accelerated by inulin and only slightly by endotoxin. Furthermore, in the presence of these reagents the clotting times of normal and C6D human blood were comparable. Similarly, inulin and endotoxin had no appreciable effect on the rate of prothrombin consumption in either normal or C6D human blood (Figs. 4 and 5).

DISCUSSION

Several investigations have suggested that complement may participate in the in vitro coagulation of normal rabbit blood (4, 5), and the recent studies of Zimmerman, Arroyave, and Müller-Eberhard (7, 8) demon-

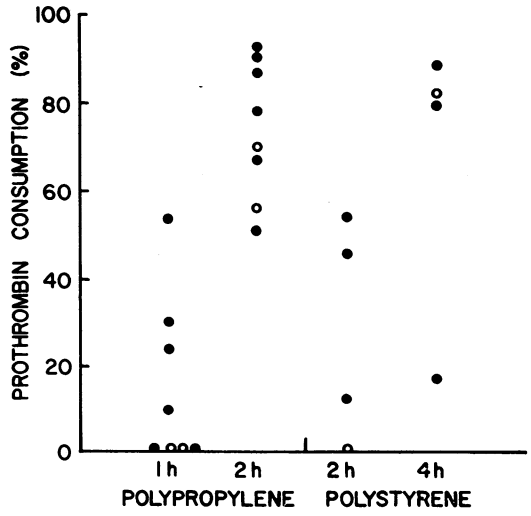

FIGURE 2 Prothrombin consumption in polypropylene and polystyrene plastic tubes at 37°C. ●, normals; ○, C6D.

strated that the rate of coagulation of blood from rabbits genetically deficient in C6 is retarded. Complement activation appears to accelerate the coagulation of rabbit blood by participating in the initiation of the platelet release reaction (22, 23) by which phospholipid coagulant activity (platelet Factor III) is released into the plasma along with other platelet constituents.

In the present study, the hemostatic functions of a human subject homozygous for C6 deficiency and lacking detectable C6 by functional and immunoprecipitin assays (9) have been found to be within normal limits. The data suggest that, in man, C6 or later complement components do not contribute significantly to normal hemostasis.

There are, however, important species differences with regard to the effects of complement on platelets which appear to correlate with the observed differences in the hemostatic functions of C6D rabbit and C6D human blood. Mueller-Eckhart and Lüscher have demonstrated that endotoxin does not induce the release reaction in human platelets or accelerate the coagulation of whole human blood, even in the presence of complement (24). In contrast, the effects of endotoxin on rabbit platelets are both profound and complement-dependent (23). In agreement with these observations the present study shows that endotoxin and inulin, in concentrations which clearly activated the complement system, had little effect on the coagulation of normal human blood. The lack of effect of these complement-activating agents on C6D human blood is, therefore, not unexpected. These observations suggest that the apparent discrepancy between C6D human and C6D rabbit blood is, in reality, a reflection of differences between normals of both species, presumably relating to platelet responsiveness to activated complement.

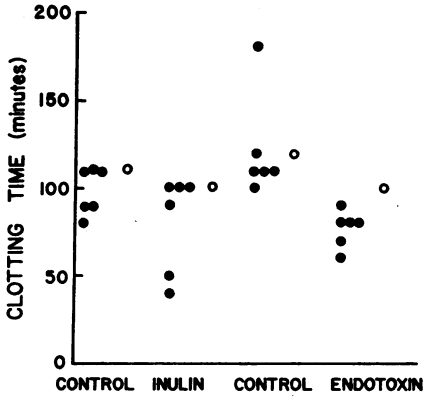

FIGURE 3 Effect of inulin (1 mg/ml) and endotoxin (40 μg/ml) on whole blood clotting time of normal and C6D human blood (polypropylene, 25°C). Concurrent control samples received equal volumes of diluent instead of inulin or endotoxin. ●, normals; ○, C6D.

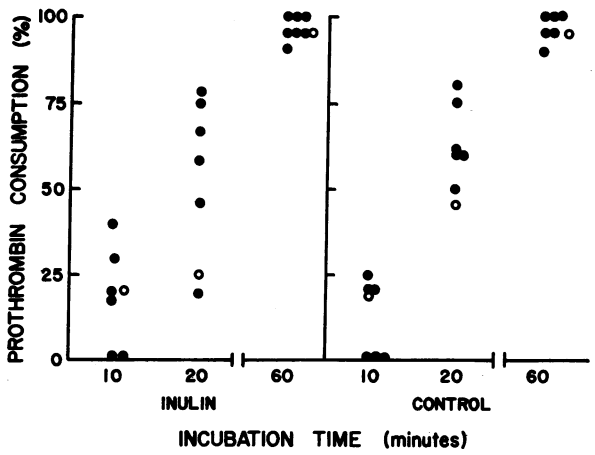

FIGURE 4 Effect of inulin (2 mg/ml) on prothrombin consumption in normal and C6D human blood (polypropylene, 37°C). Concurrent control samples received an equal volume of diluent instead of inulin. ●, normals; ○, C6D.

Zymosan, another activator of the alternate complement pathway, has been reported by Zucker and Grant to induce the release reaction in human platelets (25). Recently, Zucker [4] has found that C6D plasma from our patient effectively supports this reaction, an effect which was not enhanced by addition of functionally pure human C6 (Cordis). Whether the effect of zymosan on human platelets differs quantitatively or qualitatively from that of endotoxin or inulin appears to warrant further study.

It may be noted (Figs. 1 and 2) that in plastic tubes, the C6D human blood consistently exhibited clotting times and prothrombin consumption rates at the

[4] Dr. Marjorie B. Zucker, personal communication.

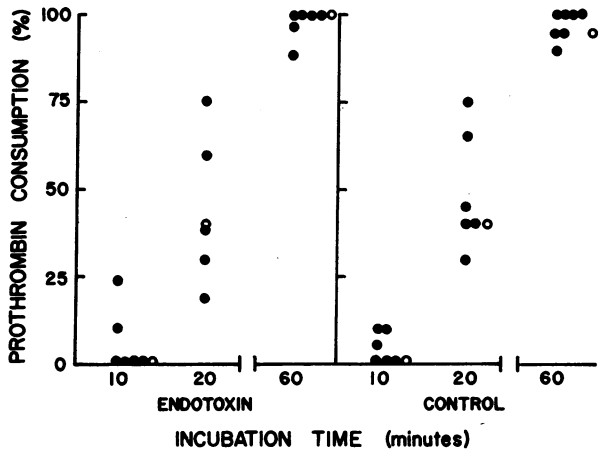

FIGURE 5 Effect of endotoxin (40 μg/ml) on prothrombin consumption in normal and C6D human blood (polypropylene, 37°C). Concurrent control samples received an equal volume of diluent instead of endotoxin. ●, normals; ○, C6D.

upper and lower limits, respectively, of the normal range. It is conceivable, therefore, that if more C6D humans could be tested, their values might all be clustered at the extreme end of the normal range. Such a finding could indicate a statistically separate subpopulation and would imply a very subtle contribution of C6 (or later complement components) to these in vitro assays. Nevertheless, from the presently available data it seems clear that absence of C6 is not accompanied by a significant defect in hemostatic function in man. These observations do not rule against a possible contribution of earlier acting human complement components (C1–C5) to normal hemostasis or against participation of complement in abnormal coagulation in certain clinical settings.

ACKNOWLEDGMENTS

The authors thank Juliann Brown, Maggie Hendrickson, and Patricia Thiem for technical assistance and Jo Ann Burns for preparation of the manuscript.

This research was supported in part by research and training grants from the U. S. Public Health Service: AM-09810 and HL-06241.

REFERENCES

1. Donaldson, V. H. 1970. Blood coagulation and related plasma enzymes in inflammation. *Ser. Haematol.* **3**: 39.
2. Ratnoff, O. D. 1969. Some relationships among hemostasis, fibrinolytic phenomena, immunity, and the inflammatory response. *Adv. Immunol.* **10**: 145.
3. Packham, M. A., and J. F. Mustard. 1971. Platelet reactions. *Semin. Hematol.* **8**: 30.
4. Horowitz, H. I., R. M. Des Prez, and E. W. Hook. 1962. Effects of bacterial endotoxin on rabbit platelets. II. Enhancement of platelet factor 3 activity in vitro and in vivo. *J. Exp. Med.* **116**: 619.
5. Robbins, J., and C. A. Stetson, Jr. 1959. An effect of antigen-antibody interaction on blood coagulation. *J. Exp. Med.* **109**: 1.
6. McKay, D. G., S. S. Shapiro, and J. N. Shanberge. 1958. Alterations in the blood coagulation system induced by bacterial endotoxins. II. In vitro. *J. Exp. Med.* **107**: 369.
7. 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.
8. Zimmerman, T. S., and H. J. Müller-Eberhard. 1971. Blood coagulation initiation by a complement mediated pathway. *J. Exp. Med.* **134**: 1601.
9. Leddy, J. P., M. M. Frank, T. Gaither, J. Baum, and M. R. Klemperer. 1974. Hereditary deficiency of the sixth component of complement in man. I. Immunochemical, biologic, and family studies. *J. Clin. Invest.* **53**: 544.
10. Breckenridge, R. T., and O. D. Ratnoff. 1962. Studies on the nature of the circulating anticoagulant directed against antihemophilic factor: with notes on an assay for antihemophilic factor. *Blood J. Hematol.* **20**: 137.
11. Quick, A. J., and J. E. Favre-Gilly. 1949. The prothrombin consumption test: its clinical and theoretic implication. *Blood J. Hematol.* **4**: 1281.
12. Pechet, L. 1964. One-stage specific determination of Factor II (Prothrombin). *In* Blood Coagulation, Hemorrhage and Thrombosis. L. M. Tocantins and L. A. Kazal, editors. Grune & Stratton, Inc., New York. 144.
13. Bleyer, W. A., and R. T. Breckenridge. 1970. Studies on the detection of adverse drug reactions in the newborn. II. The effects of prenatal aspirin on newborn hemostasis. *J. Am. Med. Assoc.* **213**: 2049.
14. Hardisty, R. M., and G. I. C. Ingram. 1965. Bleeding Disorders. Blackwell Scientific Publications Ltd., Oxford, England. 271.
15. Spaet, T. H., and J. Cintron. 1965. Studies on platelet factor-3 availability. *Br. J. Haematol.* **11**: 269.
16. Ingram, G. I. C. 1952. The determination of plasma fibrinogen by the clot-weight method. *Biochem. J.* **51**: 583.
17. Losowsky, M. S., M. D. Leeds, R. Hall, and W. Goldie. 1965. Congenital deficiency of fibrin-stabilizing factor. A report of three unrelated cases. *Lancet.* **2**: 156.
18. Ratnoff, O. D., E. W. Davie, and D. L. Mallett. 1961. Studies on the action of Hageman factor: evidence that activated Hageman factor in turn activates plasma thromboplastin antecedent. *J. Clin. Invest.* **40**: 803.
19. Langdell, R. D., R. H. Wagner, and K. M. Brinkhous. 1953. Effect of antihemophilic factor on one-stage clotting tests. A presumptive test for hemophilia and a simple one-stage antihemophilic factor assay procedure. *J. Lab. Clin. Med.* **41**: 637.
20. Quick, A. J. 1948. The determination of prothrombin by the method of Quick. Transactions of the 1st Conference on Blood Clotting and Allied Problems. Josiah Macy, Jr. Foundation. 170.
21. Bell, D. A., P. F. Leblond, J. P. Leddy, P. K. Lauf, P. L. LaCelle, and R. I. Weed. 1972. Immune hemolysis. I. Differing susceptibility of three genetic types of sheep red cells. *J. Immunol.* **108**: 467.
22. Zimmerman, T. S., and H. J. Müller-Eberhard. 1972. Interaction of complement, platelets and the blood coagulation systems. *J. Clin. Invest.* **51**: 107 *a*. (Abstr.)
23. Siraganian, R. P. 1972. Platelet requirement in the interaction of the complement and clotting systems. *Nat. New Biol.* **239**: 208.
24. Mueller-Eckhardt, C., and E. F. Lüscher. 1968. Immune reactions of human blood platelets. IV. Investigations on the problem of an immunologically induced effect of endotoxin on human platelets. *Thromb. Diath. Haemorrh.* **20**: 336.
25. Zucker, M. B., and R. Grant. 1972. Release reaction of human platelets induced by zymosan. *Fed. Proc.* **31**: 267. (Abstr.)