Proc. Natl. Acad. Sci. USA
Vol. 74, No. 2, pp. 759–763, February 1977
Medical Sciences

Macromolecular Factor VIII complex: Functional and structural heterogeneity observed in von Willebrand swine with transfusion

(hemostasis/antihemophilic factor/platelet aggregating factor/gel chromatography/density gradient ultracentrifugation)

THOMAS R. GRIGGS, JACK POTTER, S. B. MCCLANAHAN, W. P. WEBSTER, AND K. M. BRINKHOUS

Department of Pathology, University of North Carolina, Chapel Hill, N.C. 27514

Contributed by K. M. Brinkhous, November 15, 1976

ABSTRACT **The physiologic activities concerned with hemostasis and associated with the Factor VIII macromolecular complex were investigated in swine with von Willebrand's disease after infusions of cryoprecipitate, a lyophilized Factor VIII concentrate, or porcine serum. Immediately after each infusion the various activities, antihemophilic factor, von Willebrand platelet aggregating factor, and Factor VIII-related antigen, were elevated in approximate proportion to dose and the bleeding time was shortened. There was a late secondary rise in antihemophilic factor. During the period after infusion, there was a differential fall-off of the various activities, with the bleeding time effect lost first, followed by the von Willebrand platelet aggregating factor and then by the Factor VIII-related antigen. The plasma from swine with von Willebrand's disease late after infusion contained high levels of antihemophilic factor without other detectable activities of the complex. Antihemophilic factor, free of the other components, obtained from plasma from swine with von Willebrand's disease either before or late after infusion eluted from agarose gel columns both as high and lower molecular weight material, unlike normal antihemophilic factor, which had a high molecular weight. In contrast, on ultracentrifugation the antihemophilic factor in these plasmas sedimented slowly, even though chromatographically the plasmas contained both high and low molecular weight factor. All of the Factor VIII complex activities in normal porcine plasma sedimented rapidly. These studies demonstrate the heterogeneity of the Factor VIII complex and the apparent dependence of its chromatographic and sedimentation behavior on the functional activities associated with the complex.**

"Factor VIII complex" refers to a macromolecular plasma protein complex that carries several biological functions needed for hemostasis (1). These functions are concerned with both fibrin clotting and platelet plug formation. Selective functional deficiencies of the Factor VIII complex occur in hemophilia A and in von Willebrand's disease (vWD). In severe hemophilia, only the antihemophilic factor (AHF) or Factor VIII coagulant activity is lacking. In severe vWD, AHF is greatly reduced or absent, but in addition, the other activities of the Factor VIII complex are lacking. These include: (*a*) the specific platelet aggregating factor (PAF) or von Willebrand factor (vWF), which in human beings requires ristocetin for its demonstration; (*b*) the Factor VIII-related antigen, which gives a precipitin reaction with antibody raised to the Factor VIII complex; (*c*) the bleeding time factor; and (*d*) a plasma factor, different from AHF, that on transfusion of subjects with vWD causes a delayed rise in AHF.

In normal plasma and plasma concentrates, the AHF of the complex can be dissociated from the PAF/vWF (2) and the Factor VIII-related antigen (3) by an increase in the ionic strength (e.g., 0.25 M $CaCl_2$), followed by gel chromatography. The isolated AHF is of apparent low molecular weight (4), whereas the platelet aggregating activity and antigen elute as high-molecular-weight materials. Further, recombination to form a fully functional macromolecular complex has been effected by mixing the large and small molecular weight materials and removing the excess ions by dialysis (5). Highly purified Factor VIII complex yields, on reduction followed by polyacrylamide gel electrophoresis, a subunit without functional activity and of low molecular weight, about 195,000 (6). These and other studies have led to various hypotheses concerning the molecular characteristics of the Factor VIII complex and its several biological activities (1). One hypothesis, based primarily on dissociation and recombination experiments, is that the AHF is a relatively small molecule bound ionically to a larger moiety which carries the other activities of the complex (5). Alternatively, it is proposed that there is a single molecular entity, not a complex, consisting of repeating subunits, either identical or nonidentical and covalently bound (7).

Dissociation of Factor VIII complex activities is also observed in subjects with vWD after transfusion of plasma products. This transfusion response had been studied in both human subjects (8) and swine (9) with vWD. For example, late during the period after transfusion, when the bleeding time is long, relatively large amounts of AHF may be present without any PAF/vWF or Factor VIII-related antigen (10). This dissociation of the Factor VIII complex activities *in vivo* provides an avenue for further characterizing the complex in relation to its physiologic activities. In the studies reported here, the transfusion response was studied in swine with vWD infused with several plasma products. The behavior of AHF and other Factor VIII complex activities in the plasmas after infusion was determined by agarose gel chromatography, which should provide an indicator of molecular size, and by sucrose density gradient ultracentrifugation, which could indicate the state of aggregation or density of the complex.

MATERIALS AND METHODS

Swine with vWD. Swine with vWD were from the Chapel Hill colony of the University of Missouri Poland China strain (9). Homozygous male and female pigs, products of matings of females heterozygous and males homozygous for the bleeder state, were used in the transfusion experiments.

Factor VIII Plasma Fractions. Two different porcine plasma fractions were used for transfusion. (*a*) Cryoprecipitate was prepared by collecting normal porcine blood from the cephalic vein into acid-citrate-dextrose solution (9). The plasma was transferred to plastic "transfer packs" and ethanol was added to 8% by volume. Freezing was at −70° (24 hr) followed by thawing at 4° (24 hr). The cryoprecipitate was collected by centrifugation (3000 × *g*, 30 min, 4°) and stored at −20° until used. Immediately before use, it was dissolved in normal saline, one-tenth original plasma volume. (*b*) Lyophilized Factor VIII concentrate (AHG, Speywood, Inc., Nottingham, U.K., supplied

Abbreviations: vWD, von Willebrand disease; PAF, platelet aggregating factor; vWF, von Willebrand factor; AHF, antihemophilic factor.

through the courtesy of Mr. D. Heath) was dissolved in normal saline for use. Porcine serum, containing approximately 20% AHF and 100% PAF, was also infused in one experiment.

Transfusion Experiments. The Factor VIII concentrates were infused into the cephalic vein of swine with vWD through a 14-gauge plastic catheter with attached plastic stopcock. Injection time was approximately 5 min. The catheter remained inserted for about 2 hr; the patency was maintained by slow infusion of Ringer's lactate solution. Blood samples for preparation of citrated plasma were obtained immediately before infusion, and usually at 15 min, and 1, 2, 6, 12, 24, 48, 72, 96, and 120 hr after infusion. The first four samples were collected from the catheter; thereafter, blood was sampled with an 18-gauge needle. Citrated plasma samples were stored at −70° in 0.5-ml aliquots.

Assays for Factor VIII Complex Activities. (*a*) PAF was assayed by a modification of the macroscopic aggregation method of Griggs *et al.* (9), in which formaldehyde-fixed human platelets prepared by a modification of the method of Allain *et al.* were used (11). The test system consisted of 0.2 ml of platelet suspension and 0.1 ml of diluted test materials. The assay was sensitive to <0.05 units/ml of PAF. One unit of PAF is that amount contained in 1 ml of pooled normal porcine plasma. (*b*) AHF was assayed by a modification of the partial thromboplastin time assay of Langdell and coworkers (9, 12), using canine hemophilic substrate. The assay was sensitive to <0.0125 unit of AHF per ml (e.g., clotting times: blank >70 sec; 0.0125 unit of AHF per ml, 60 sec). In some experiments, human hemophilic plasma was used as substrate. Specificity of the assay for AHF was demonstrated by neutralization tests in which two parts test material were mixed with one part diluted plasma from a patient with hemophilia with a high titer of inhibitor. (*c*) Factor VIII-related antigen was determined by a modification of the quantitative electrophoretic method of Zimmerman *et al.* (13). Rabbit antibody against porcine Factor VIII complex was raised by injection of the void volume fraction of the Speywood porcine concentrate. For chromatography, Biogel A-15 m was used (30 × 2.5 cm column at 25°, flow of 8 ml/cm^2 per hr). Elution was with Tris-buffered saline (0.05 M Tris·HCl, 0.15 M NaCl, pH 7.35). The void volume fraction contained 2.5 units/ml of AHF and 5.5 units/ml of PAF. New Zealand white rabbits were injected with 0.2 ml subcutaneously, then with 0.1 ml at weekly intervals for 3 weeks, and again at 12 and 13 weeks. Blood was collected by cardiac puncture and allowed to clot for 24 hr. The serum was then harvested. Control plasma for this assay as well as for the PAF and AHF assays was from a pool of five normal animals. (*d*) Saline bleeding times were determined by the method of Mertz (14).

Chromatographic Studies. Column chromatography was performed with 4% agarose (Biogel A-15 m, 200–400 mesh, Bio-Rad Laboratories, Richmond, Calif). Columns were siliconized glass, 0.9 × 23 cm. Elution buffer was 0.05 M Tris·HCl, pH 7.2. Sample size was 0.5 ml, fractions were collected in 0.5-ml aliquots, and the flow rate was 8 ml/cm^2 per hr at 25°. Material was eluted from the columns downward by gravity. Samples were assayed for AHF, PAF, and Factor VIII-related antigen.

Ultracentrifugal Studies. A sucrose density gradient of 10–40% was prepared in Tris-buffered saline (0.05 M Tris·HCl, 0.15 M NaCl, pH 7.2, 10–11 ml), in 13-ml cellulose nitrate tubes. The citrated plasma sample (1 ml) was layered on the top of the gradient with a pasteur pipette. The tube was then centrifuged in a Beckman L3-50 ultracentrifuge in a swinging bucket head (SW-36, Beckman Instruments) at 36,000 rpm or an average of 140,000 × *g* at 4° for 16 hr. Samples were taken from the bottom of the tube in 1-ml aliquots. Each sample was placed in a Visking tube and dialyzed against Tris-buffered saline for 12 hr at 4°. The samples were then assayed for AHF and PAF.

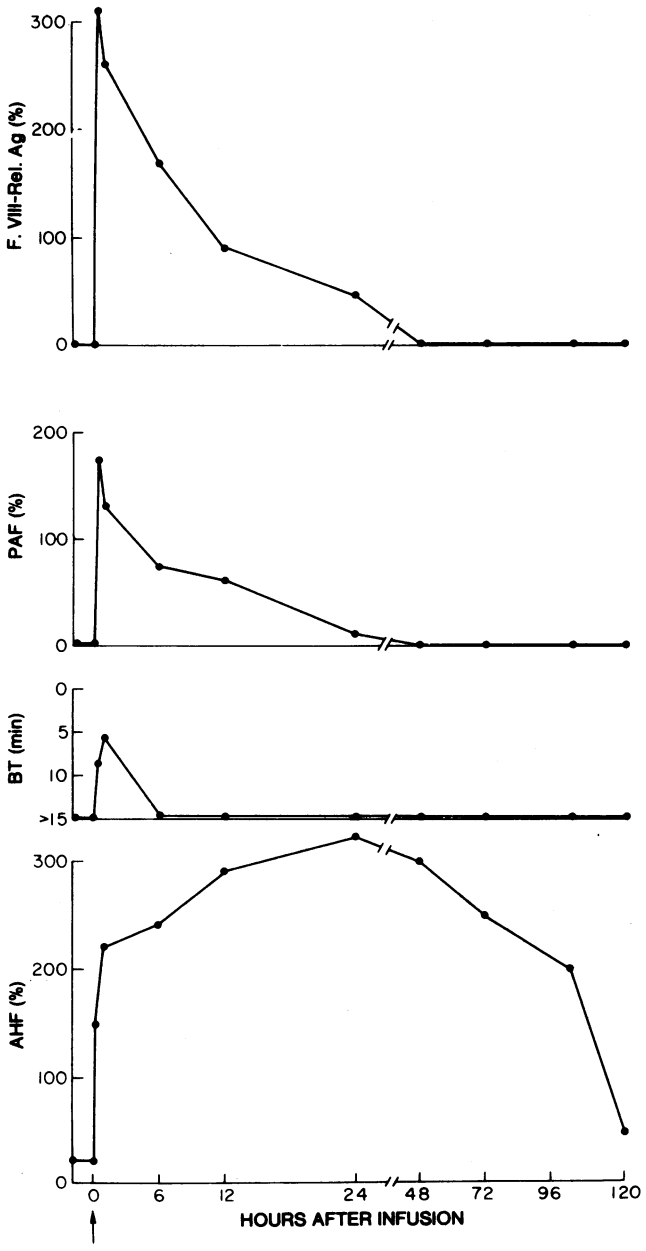

FIG. 1. Infusion of pig with vWD (female, 96 kg) with 470 ml of porcine Factor VIII concentrate containing 12.2 units/ml of AHF and 5.1 units/ml of PAF. Arrow indicates time of infusion. Serial determinations of Factor VIII complex activities—AHF, PAF, Factor VIII-related antigen, and bleeding time (BT)—were made. Baseline values on charts are <5% for PAF and Factor VIII-related antigen and <1.25% for AHF.

RESULTS

Transfusion response

Fig. 1 illustrates the typical response of the various Factor VIII complex activities after infusion of a Factor VIII concentrate into a pig with vWD. Before infusion, the AHF level was 24% and the bleeding time was greater than 15 min. Neither PAF nor the Factor VIII-related antigen could be detected. After

Table 1. Response of swine with vWD to transfusions of porcine cryoprecipitate, lyophilized concentrate, and serum

Exp. no.	Bleeder swine, kg	Before infusion				After infusion at 15–60 min				HDT*		Maximum Factor VIII without PAF or Ag†	
		Factor VIII (%)	PAF (%)	Ag† (%)	BT‡ (min)	Factor VIII (%)	PAF (%)	Ag† (%)	BT‡ (min)	PAF (%)	Ag† (%)	Time (hr)	Factor VIII (%)
Porcine cryoprecipitate infusion													
1	28	19	0	—	>15	98	46	—	5	3.0	—	24	147
2	26.5	15	0	0	>15	170	141	95	6	4.8	7.2	48	73
3	100.0	22	0	0	>15	79	188	99	—	4.3	6.8	24	90
Porcine lyophilized concentrate infusion													
4	29.0	24	0	—	>15	122	90	—	3	5.4	—	24	116
5	96.0	24	0	0	>15	220	176	315	6	3.5	7.0	48	298
6	39.5	28	0	—	>15	157	180	—	3.5	4.2	—	24	186
Porcine serum infusion													
7	25.0	17	0	—	>15	70	32	—	10	3.4	—	12	78

* HDT = half-disappearance time.
† Ag = Factor VIII-related antigen.
‡ BT = bleeding time.

infusion, there was an immediate rise in all plasma activities, and the bleeding time was shortened to the normal range for a few hours. The AHF response curve after infusion was biphasic, with the initial peak occurring immediately after injection and a delayed peak of activity occurring at 24 hr. In contrast, the PAF and Factor VIII-related antigen levels showed a single peak immediately after transfusion, followed by a rapid disappearance from plasma. Approximately half the plasma PAF had disappeared in 3.5 hr, and half the Factor VIII-related antigen in 7 hr. At 48 hr, when the AHF was 298%, no PAF or Factor VIII-related antigen could be detected. Thus, the plasma at this time contained only one of the four activities of the Factor VIII complex, AHF.

The response to transfusion in swine with vWD was studied in seven separate experiments (Table 1). Separate bleeder swine were used except for experiments 4 and 5, where the same animal was used many months apart. Before infusion, the findings were similar in all animals. PAF and Factor VIII-related antigen were undetectable, bleeding time was prolonged, and AHF values were 15–28% of normal. Cryoprecipitate, a lyophilized Factor VIII concentrate, and, in one experiment, porcine serum were infused. Plasma levels of the different Factor VIII complex activities immediately after infusion were in general dependent on the dose given. However, the actual levels of AHF, PAF, and Factor VIII-related antigen and the relationships between them were not precisely predictable based on calculations utilizing estimated plasma volume and dose infused. AHF present in the plasma 24–48 hr after infusion of the plasma fractions was often at higher levels than observed during the first hour after infusion. After the serum infusion, the plasma at 12 hr was free of PAF but had a near normal amount of AHF.

Characterization of "Factor VIII complex" after infusion

The distribution patterns of AHF on agarose gel chromatography and on sucrose density gradient ultracentrifugation were compared in plasmas collected from pigs with vWD 1, 48, and 120 hr after infusion. Normal porcine plasma and plasma from swine with vWD before infusion similarly studied served as controls. The presence or absence of PAF and Factor-VIII-related antigen associated with AHF in the "Factor VIII complex" was also determined. The findings were similar in three separate experiments. The data from one experiment (Exp. 5, Table 1) are presented in Fig. 2.

The behavior of AHF in normal plasma and plasma from swine with vWD before infusion is illustrated in the top two panels of Fig. 2. The AHF in the normal plasma, which also contained normal amounts of PAF and Factor VIII-related antigen, eluted predominantly in the void volume, and on ultracentrifugation sedimented to the higher density sucrose region near the bottom of the tube. The AHF in the plasma from swine with vWD, which was free of PAF and antigen, was distributed in a biphasic pattern on chromatography; part eluted in the void volume, comparable to AHF in normal plasma, and part eluted in later fractions. In contrast, there was only a single peak of AHF activity on ultracentrifugation; the AHF remained near the top of the tube. Normal plasma, diluted 1:4 with citrated saline to give an AHF level comparable to that in plasma from swine with vWD, was studied in a similar manner. The AHF in the diluted normal plasma was distributed like that in undiluted normal plasma, indicating that the pattern for plasma from swine with vWD was not merely a reflection of low AHF concentration.

In the plasma after infusion, shifting patterns of distribution of AHF were observed, varying with the elapsed time after transfusion and with the presence or absence of associated PAF and Factor VIII-related antigen. In the sample from swine with vWD taken *1 hr* after infusion, in which both AHF and PAF were greater than 100%, the chromatographic and ultracentrifugal patterns were similar to those of normal plasma. However, there was some trailing of AHF in late elution volumes on chromatography and in more slowly sedimenting fractions higher in the tube on ultracentrifugation.

In the sample taken *48 hr* after infusion, AHF was present in normal amounts but was completely dissociated from the other Factor VIII complex activities. On chromatography, such samples showed a unique pattern of distribution of AHF, different from that in either normal plasma or plasma from swine with vWD plasma. Although the AHF was broadly distributed

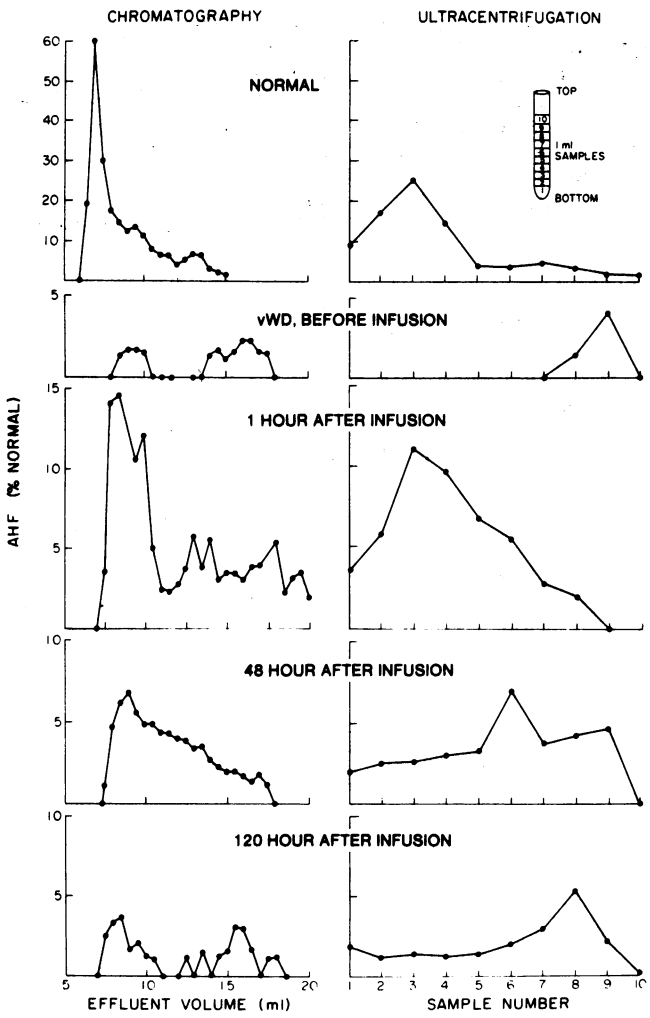

FIG. 2. Results of gel chromatography and sucrose density gradient ultracentrifugation of normal porcine plasma and serial samples of plasma from a pig with vWD before and after infusion of Factor VIII concentrate (Speywood). See legend of Fig. 1 and Exp. 5, Table 1 for details. See *Materials and Methods* for gel filtration and sedimentation procedures. Plasma applied to the chromatographic column and sedimentation tube, respectively, contained the following amounts of AHF: normal, 0.50 and 1.00 unit; before infusion, 0.12 and 0.25 unit; 1-hr sample, 1.10 and 2.20 units; 48-hr sample, 0.78 and 1.57 units; and 120-hr sample, 0.24 and 0.47 unit.

in all fractions, the void volume gave the highest assay values. On ultracentrifugation, in contrast, the AHF was mainly in the lower density region high in the tube, with samples 6–9 containing the greatest amounts.

In the sample taken *120 hr* after infusion, the concentration of AHF was approaching the range before infusion, and its distribution on both chromatography and ultracentrifugation was similar to that of control plasma from swine with vWD.

DISCUSSION

These studies extend earlier findings on the transfusion response of subjects with vWD, human (8, 15–19) and swine (9, 10, 20, 21). The three materials infused, cryoprecipitate, lyophilized Factor VIII concentrate, and porcine serum, all caused the same qualitative response, in that they all corrected the deficiencies in AHF, PAF, and hemostasis as indicated by a shortened bleeding time. Factor VIII-related antigen was also increased. All infusions produced a secondary rise in plasma AHF. Serum infusions had been shown earlier to cause the delayed rise in AHF (20), but not to correct the bleeding time. Normal plasma infused in swine (9) in amounts sufficient to increase the PAF to about 30% of normal with both an immediate and delayed rise in AHF likewise did not correct the bleeding time. These findings suggested the possibility that there is a bleeding time factor separate from PAF, or alternatively that the seeming dissociation is a dose effect. Recently it was shown that infusion of lyophilized human Factor VIII concentrates into human subjects with vWD corrected all of the vWD deficiencies except the prolonged bleeding time, even if the levels of AHF, vWF, and Factor VIII-related antigen were over 100% of normal (19). If the bleeding time factor is a distinct molecular activity, it apparently coprecipitates with PAF in the porcine plasma fractions, is stable on lyophilization, and, like PAF/vWF, is present in serum.

Differences in the rate of disappearance of the various Factor VIII complex activities with time after transfusion were striking. The bleeding time effect is lost first, then the PAF activity, followed by loss of factor VIII-related antigen activity, all within about 24 hr, dependent on dose infused. The most striking dissociation of the Factor VIII complex activities was seen later, during the secondary rise in AHF. The AHF level was at normal or supernormal levels (78–298%, Table 1) but devoid of other activities of the complex. The difference in fall-off of PAF and Factor VIII-related antigen suggests that they are not simply expressions of the same molecular entity. The antigen in normal plasma appears to be heterogeneous when examined immunochemically, but the physiological attributes of the different normal antigens are unclear (22).

The factor stimulating the delayed rise in AHF, while observed in all of the Factor VIII materials infused in this study, was not tested for in the plasma from swine with vWD after infusion. The results of one experiment by Bowie *et al.* (18) suggest that this activity is not present in plasma from swine with vWD 6 hr after infusion. Data are insufficient to indicate its identity or nonidentity with the other non-AHF activities, however, or its rate of disappearance after infusion.

AHF devoid of the specific vWD group of activities can be obtained with this experimental swine model at any level of AHF desired, from 15–25% in the untransfused swine with vWD to as high as 300% of normal after infusion. In each case, the AHF appears as both high and low molecular weight material, as judged by gel filtration chromatography. Although this suggests two populations of AHF in plasma from swine with vWD, the results of the sedimentation experiments failed to show two distinct sedimentation patterns, fast and slow. AHF associated with the other activities of the normal complex sedimented rapidly to the bottom of the tube, whereas the AHF in plasma from swine with vWD sedimented more slowly. Apparently the altered composition of this "AHF-only" molecular complex was more important than its size in determining its sedimentation characteristics. Perhaps relatively different carbohydrate or water content of the complex could account for the findings. It was surprising nevertheless not to find a fast sedimenting component correlating with the void volume material on gel chromatography. This suggests that the "AHF-only" macromolecular complex may be aggregated AHF molecules or a complex with a modified carrier protein that had lost its vW functional activities, as judged by the bioassays used. It may even be a complex with another protein(s). It would appear that manipulation of the ratios of Factor VIII activities in this model and analysis of the spectrum of macromolecular complexes obtained provide additional insights into the heterogeneity of this still enigmatic complex and the kinetics of its assembly.

We are indebted to Roger Benson, Albany, N.Y., for supplying some of the rabbit antibody. Supported in part by NIH Grants HL-01648 and HL-06350, and North Carolina Heart Association Grant 74-A-52.

1. Cooper, H. A., Mason, R. G. & Brinkhous, K. M. (1976) *Annu. Rev. Physiol.* **38**, 501–535.
2. Griggs, T. R., Cooper, H. A., Webster, W. P., Wagner, R. H. & Brinkhous, K. M. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2814–2818.
3. Rick, M. E. & Hoyer, L. W. (1973) *Blood* **42**, 737–747.
4. Owen, W. G. & Wagner, R. H. (1972) *Thromb. Diath. Haemorrh.* **27**, 502–515.
5. Cooper, H. A., Griggs, T. R. & Wagner, R. H. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2326–2329.
6. Shapiro, G. A., Anderson, J. C., Pizzo, S. V. & McKee, P. A. (1973) *J. Clin. Invest.* **52**, 2198–2210.
7. Switzer, M. E. & McKee, P. A. (1976) *J. Clin. Invest.* **57**, 925–937.
8. Bennett, B., Ratnoff, O. D. & Levin, J. (1972) *J. Clin. Invest.* **51**, 2597–2601.
9. Griggs, T. R., Webster, W. P., Cooper, H. A., Wagner, R. H. & Brinkhous, K. M. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2087–2090.
10. Griggs, T. R., Potter, J., McClanahan, S., Webster, W. P. & Brinkhous, K. M. (1976) *Fed. Proc.* **35**, 730.
11. Allain, J. P., Cooper, H. A., Wagner, R. H. & Brinkhous, K. M. (1975) *J. Lab. Clin. Med.* **85**, 318–328.
12. Langdell, R. D., Wagner, R. H. & Brinkhous, K. M. (1953) *J. Lab. Clin. Med.* **41**, 637–647.
13. Zimmerman, T. S., Ratnoff, O. D. & Powell, A. E. (1971) *J. Clin. Invest.* **50**, 244–254.
14. Mertz, E. T. (1942) *Am. J. Physiol.* **136**, 360–362.
15. Bloom, A. L., Peake, I. R. & Giddings, J. C. (1973) *Thromb. Res.* **3**, 389–404.
16. Muntz, R. H., Ekert, H. & Helliger, H. (1974) *Thromb. Res.* **5**, 111–123.
17. Kernoff, P. B. A., Rizza, C. R. & Kaelin, A. C. (1974) *Br. J. Haematol.* **28**, 357–370.
18. Bowie, E. J. W., Fass, D. N., Olson, J. D. & Owen, C. A. (1974) *Thromb. Res.* **5**, 479–494.
19. Blatt, P. M., Brinkhous, K. M., Culp, H. R., Krauss, J. S. & Roberts H. R. (1976) *J. Am. Med. Assoc.* **236**, 2770–2772.
20. Muhrer, M. E., Lechler, E., Cornell, C. N. & Kirkland, J. L. (1965) *Am. J. Physiol.* **208**, 508–510.
21. Chan, J. Y. S., Owen, C. A., Bowie, E. J. W., Didisheim, P., Thompson, J. H., Muhrer, M. E. & Zollman, P. E. (1968) *Am. J. Physiol.* **214**, 1219–1224.
22. Zimmerman, T. S., Roberts, J. & Edgington, T. S. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 5121–5125.