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Research Article

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Factor V Anticoagulants: Clinical, Biochemical, and Immunological Observations

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ABSTRACT A patient who had received multiple transfusions for complications of acute hemorrhagic pancreatitis developed a potent factor V anticoagulant with bleeding due to defective hemostasis. Despite its potency, the anticoagulant disappeared within 15 days of its first manifestation. A second patient with adenocarcinoma of the colon developed an anticoagulant to factor V postoperatively after a single blood transfusion. The anticoagulants appeared to react stoichiometrically with factor V in normal plasma in vitro. They had the physicochemical properties of immunoglobulins, and their activity was neutralized by antihuman immunoglobulin antiserum. One anticoagulant appeared to be slightly more active against homologous than against autologous factor V, but it also inhibited heterologous factor V. Both anticoagulants progressively inactivated intrinsic prothrombin activator formed from normal reagents in the incubation mixture of the thromboplastin generation test, thus confirming that factor V is required for the effective action of the intrinsic prothrombin activator. Since the anticoagulants were immunoglobulins whose activity was consumed in their reaction with factor V, consumption of anticoagulant activity was used to detect factor V antigenic material in test materials. Human serum without factor V clotting activity was found to consume anticoagulant activity, i.e., to contain inactive factor V antigenic material. Plasma from two patients with hereditary factor V deficiency (parahemophilia) failed to consume significant anticoagulant activity. Thus, the lack of factor V activity in these patients represents a deficiency of factor V molecules rather than the synthesis of a defective molecule with impaired clotting activity.

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INTRODUCTION

Acquired anticoagulants are a well-documented cause of hemorrhagic disease. One relatively common anticoagulant acts against factor VIII (antihemophilic globulin) and has been characterized as an immunoglobulin. It has been found in patients with hereditary factor VIII deficiency after transfusions, in postpartum women, in disorders characterized by immunologic phenomena, e.g. in systemic lupus erythematosus, rheumatoid arthritis, and drug reactions, and in elderly patients without underlying disease.

This paper is a report of studies on a much rarer anticoagulant, a potent inhibitor of factor V (proaccelerin), which was found in two patients. The mechanism of action and the properties of each patient's inhibitor were characterized. These inhibitors were used to study further the role of factor V in clotting and to define the defect in two patients with hereditary factor V deficiency (parahemophilia) (1).

METHODS

Clotting methods

Platelet-poor plasma was prepared by high speed centrifugation of 9 volumes of venous blood added to 1 volume of a balanced citrate anticoagulant (2). Plastic syringes and tubes were used. Serum was obtained by clotting blood in glass tubes and incubating the clotted blood for 2 hr at 37°C. Plasma and serum were used either fresh or after storage at −20°C in capped, plastic vials. Citrated saline was made by adding one part of 0.1 M trisodium citrate to five parts of 0.15 M sodium chloride, as described elsewhere (2).

Factor V was measured in a one-stage system in which 0.1 ml of human brain thromboplastin (3), of either hereditary factor V deficiency plasma or normal plasma depleted of factor V (4), and of test substance diluted in citrated saline were incubated together for 3 min at 37°C and then clotted by adding 0.1 ml of 40 mM calcium chloride solution.

Modified thromboplastin generation test systems were utilized for some experiments. Citrated plasma was adsorbed with aluminum hydroxide gel as described previously (2),

and a cephalin reagent (5) was used in place of platelets. Details of these test systems are given in the Results section.

The following techniques were used in this laboratory for other clotting tests and have been described elsewhere: Quick prothrombin time (6), prothrombin time by the P and P method (6), activated partial thromboplastin time (2), factor II (prothrombin) (5) modified by adding a serum reagent to supply factor X (Stuart-Prower factor), factor VII (proconvertin) (7), factor VIII (8), factor IX (plasma thromboplastin component) (9), factor X (10), factor XI (plasma thromboplastin antecedent) (11), and factor XII (Hageman factor) (12). Factor XIII was assayed qualitatively by observation of a fibrin clot incubated in 5 M urea at room temperature for 24 hr.

Clotting times were converted to per cent activity from dilution curves made with a pooled plasma from 11 normal subjects. The normal pooled plasma was stored at −20°C.

Immunological methods

Starch zone electrophoresis was performed by the technique of Kunkel (13); chromatography was performed on DEAE (diethylaminoethyl)-cellulose by the technique of Fahey and Horbett (14); and gel filtration was done on Sephadex G-200 (90 × 2.5 cm column) by the technique of Flodin and Killander (15). IgG was digested with papain, and the Fab and Fc fragments were separated by chromatography on DEAE-cellulose as described by Franklin (16). Protein concentration was determined either by a modified Folin technique (17) or by measuring absorbancy at 280 mμ. Antigens were emulsified with an equal volume of complete Freund's adjuvant to give a final protein concentration of 2.5 mg/ml.

Antihuman immunoglobulin antiserum was prepared by hyperimmunizing rabbits to human Cohn fraction II which had been purified by passage through a DEAE column equilibrated with a phosphate buffer at pH 8.0 and ionic strength 0.01. The antiserum reacted strongly with κ and λ light-chains and with γ chains. It reacted only very weakly with α chains and not at all with μ chains. Since it reacted with both antigenic types of light chains, it reacted with all classes of human immunoglobulins and was, therefore, referred to as antihuman immunoglobulin antiserum.

Antiserum specific for IgG was made by absorbing the above antiserum with purified Fab fragment which had been prepared by papain digestion of purified Cohn fraction II.

Antiserum specific for IgM was made by hyperimmunizing rabbits with a macroglobulin purified from a patient with macroglobulinemia of Waldenström. The patient's serum was dialyzed against distilled water; the resultant precipitate was dissolved in isotonic saline and applied to a Sephadex G-200 column equilibrated with 0.3 M NaCl; and the eluate from the first half of the first peak was concentrated by negative pressure in dialysis casing. This material gave a single line on immunoelectrophoresis with rabbit antihuman serum. The antiserum obtained with this antigen was absorbed with umbilical cord sera to make it specific for μ chains.

Antiserum specific for IgA was prepared by hyperimmunizing rabbits with an IgA myeloma protein isolated from the serum of a patient with multiple myeloma. The protein was isolated by starch block electrophoresis and further purified by column chromatography on Sephadex G-200. Eluates from the first half of the first peak were combined and concentrated. This material contained less than 1% IgG. The antiserum obtained with this antigen was absorbed with umbilical cord sera to make it specific for α chains.

Antisera specific for κ and λ light-chains were prepared by hyperimmunizing rabbits to κ and λ Bence Jones proteins. These proteins were isolated from the urine of patients with multiple myeloma by mixing 3 volumes of urine with 2 volumes of 100% saturated ammonium sulfate, by dissolving the precipitate in 0.15 M NaCl, by dialyzing extensively against distilled water, and by further purifying by starch-zone electrophoresis. The antisera were made specific by absorption with the opposite antigenic type of light chain and with Fc fragment.

Clotting factor activities were removed from antisera and from normal rabbit serum as follows: one part of citrate anticoagulant was added to five parts of serum; the serum was heated at 56°C for 30 min; and the heated serum was then adsorbed with aluminum hydroxide gel (Cutter Laboratories, Berkeley, Calif.) diluted 1:4 with distilled water (0.1 ml of gel per ml of serum). After such treatment all sera were devoid of significant clotting factor activities.

To interpret neutralization experiments one must have defined the zone of antigen and antibody excess for the reaction mixtures, which in our experiments were mixtures of the immunoglobulin antisera and the patient's plasma containing the inhibitor. Therefore, the patient's plasma was diluted in increasing amounts of each immunoglobulin antisera, incubated at 37°C for 1 hr, centrifuged, and the supernatant of each mixture was reacted in double diffusion in agar against the patient's plasma (antigen) and the antiserum (antibody). If the supernatant reacted with the patient's plasma but not with the antiserum, then the original dilution was in antibody excess. If the supernatant reacted with the antiserum but not with the patient's plasma, then the dilution was in antigen excess. If the supernatant reacted with neither the patient's plasma nor the antiserum, then the dilution was near the equivalence point.

CASE REPORTS

Patient No. 1. H. G. (No. 266-26-84), a 36 yr old Negro man, entered the Los Angeles County-University of Southern California Medical Center on August 10, 1966 because of abdominal pain, intermittent vomiting, diarrhea for 2½ months, and an increase in symptoms for 10 days. He had a long history of alcoholism. He denied any previous bleeding history. A tender epigastric mass was found on physical examination. A urine diastase was 14,500 Somogyi units (N < 500).

He received isotonic electrolyte solutions, plasma, and blood. Because of continuing fever and arteriographic findings consistent with a pseudocyst of the head of the pancreas, he underwent exploratory surgery on September 2. A pancreatic pseudocyst was drained externally, and a gastroenterostomy was performed. The estimated blood loss was 700 ml. He received 1 U of blood during surgery.

The wound edges did not bleed excessively in the postoperative period. However, 4 days postoperatively he bled massively from the upper gastrointestinal tract. He was reoperated upon on September 8, and a bleeding artery at the gastroenterostomy site was ligated. Again, hemostasis at surgery was easily achieved, and excessive postoperative oozing was not noted. As a result of the

complications described above, he received a total of 12 U of plasma and 20 U of whole blood between August 10 and September 14.

Bleeding attributable to a hemorrhagic disorder was first noted on September 14 when a large hematoma formed at the site of insertion of a femoral vein catheter. Surgical exploration disclosed only generalized oozing. On September 16 a long clotting time was discovered, and the clotting studies, which will be described, revealed an anticoagulant against factor V. The anticoagulant was still present in blood drawn on September 23, but it was no longer present in blood drawn on September 29. Between September 13 and September 29, the patient received 20–80 U of ACTH gel daily. He had many other complications, but he slowly recovered and was eventually discharged without sequelae.

Patient No. 2. E. G., a 65 year old Caucasian male, entered the Huntington Memorial Hospital, Pasadena, California on April 30, 1968 with complete obstruction of the transverse colon. His left colon had been resected in 1964 because of an adenocarcinoma. He had not bled excessively after this surgery. On May 2 he underwent a resection of the transverse colon, again without excessive bleeding. He received one blood transfusion postoperatively. Also, postoperatively, he developed transient mild jaundice of unknown cause. On May 16, 1968 he was reoperated because of a wound dehiscence. He did not bleed excessively during this procedure, but on May 17 he developed hematochezia, and on May 20 his Quick prothrombin time was discovered to be less than 10% of normal. Tests on May 25 (see Table I) established the presence of a factor V anticoagulant. The hematochezia continued during the remainder of his hospital course, and he received 18 transfusions. After discovery of the factor V anticoagulant, he was transfused only with concentrated red blood cells and albumin. Despite the anticoagulant, excessive bleeding was not noted around the abdominal wound. On June 9 his condition deteriorated rapidly, and he died. An autopsy revealed a large hematoma underlying the surgical wound and extending to the peritoneal surface. The bowel wall was necrotic at the site of the anastomosis. Other findings included a small left subhepatic abscess and a metastatic nodule of adenocarcinoma in the lung.

RESULTS

Initial clotting studies. The results of our screening clotting tests on these patients are summarized in the upper part of Table I. The marked prolongation of both the partial thromboplastin time and the Quick prothrombin time and the failure of normal plasma to correct both tests indicated that an anticoagulant was interfering with both intrinsic and extrinsic clotting. Since factor V deficiency does not affect the prothrombin time by the P and P method, its relatively normal value, despite the greatly prolonged Quick prothrombin time, suggested that these patients' plasmas lacked factor V activity.

TABLE I
Initial Clotting Studies

	Patient No. 1	Patient No. 2
Screening tests		
Partial thromboplastin time		
Patient	180 sec	180 sec
Normal	47 sec	41 sec
Patient:normal, 1:1	94 sec	73 sec
Quick prothrombin time		
Patient	90 sec	110 sec
Normal	16 sec	15 sec
Patient:normal, 1:1	48 sec	71 sec
Prothrombin (P and P method)		
Patient	62%	85%
Normal	100%	100%
Thrombin time		
Patient	37 sec	42 sec
Normal	30 sec	28 sec
Specific assays		
Factor I	—	400 mg/100 ml
Factor II	68%	78%
Factor V	0%*	0%*
Factor VII	83%	100%
Factor VIII	86%	85%
Factor IX	100%	105%
Factor X	66%	72%
Factor XI	40%	144%
Factor XII	28%	72%
Factor XIII	Normal	—

* Uncorrected by mixing equal parts of normal plasma and patient's plasma.

The values for specific clotting factor assays are listed in the bottom part of Table I. The absence of factor V activity in the patients' plasmas and in a mixture of the patients' plasmas and normal plasma proved that an anticoagulant against factor V was present. The moderate decreases in factor XI and factor XII in patient No. 1 could be interpreted either as a mild decrease of these factors or as interference by the factor V inhibitor with the assay systems.

Specificity of the inhibitors for factor V. Normal plasma was mixed at room temperature with an equal volume of that dilution (in citrated saline) of each patient's plasma that gave a relatively normal activity in each clotting factor assay before incubation (30 sec. after mixing). 30 min later subsamples were removed and assayed again for clotting factor activities. Only

factor V activity significantly decreased over the 30 min incubation period. The remaining clotting factors either remained unchanged or fell only very slightly in the 30 min sample. A control mixture of normal plasma and citrated saline did not lose factor V activity after standing for 30 min. This experiment established that each patient's inhibitor was specific for factor V.

The reaction between increasing dilutions of the anticoagulant and factor V over time. Increasing dilutions of each patient's plasma were incubated at room temperature with normal plasma; and at ½, 15, and 30 min, subsamples were assayed for factor V activity (Fig. 1). Inhibition was demonstrable at ½ min only at a high concentration of the inhibitors. Inhibition was demonstrable at 15 min in all but the most dilute concentrations tested; further inhibition was not observed at 30 min. The titer of the inhibitor in patient No. 1 was 1:20, and in patient No. 2 it was 1:40. Apparently, the inhibitors were consumed in a nonenzymatic reaction. The inverse relationship between residual factor V activity at 30 min and inhibitor concentration strongly suggested that the reaction was stoichiometric.

Physicochemical properties of the anticoagulants. The anticoagulants were as potent in serum as in plasma and were not removed from plasma by adsorption with aluminum hydroxide gel or dialysis across a semipermeable membrane (dialysis tubing; Union Carbide Corporation, New York.) for 24 hr at 4°C. Their activity was unaffected by incubation at 37°C for 24 hr or by heating at 56°C for 30 min, but it was destroyed by boiling for 5 min. The anticoagulants were found in the γ region on starch-zone electrophoresis (Fig. 2) and in the second peak on Sephadex G-200 chromatography (Fig. 3). All of these physicochemical properties suggested that the anticoagulants were immunoglobulins.

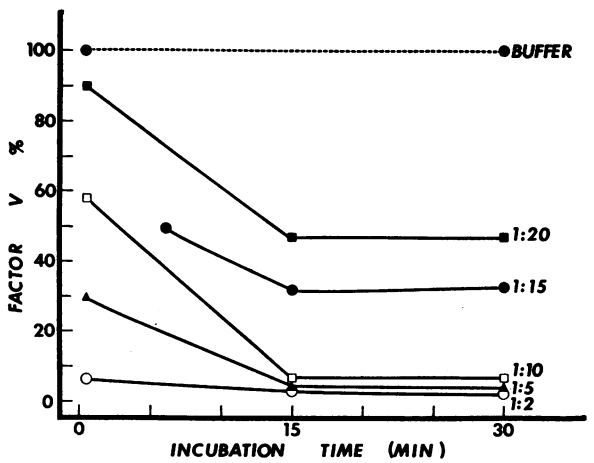

FIGURE 1 Factor V activity in normal plasma incubated with increasing dilutions of patient No. 1's plasma. Equal parts of normal plasma and either citrated saline, as a control, or the dilutions of patient No. 1's plasma shown in the figure were incubated together at room temperature. At the times shown, subsamples were removed, diluted 1:5 in citrated saline, and assayed for factor V activity. In this and subsequent figures the ordinate labeled "Factor V %" refers to factor V, per cent initial concentration. (The same experiment done with patient No. 2's plasma yielded similar findings.)

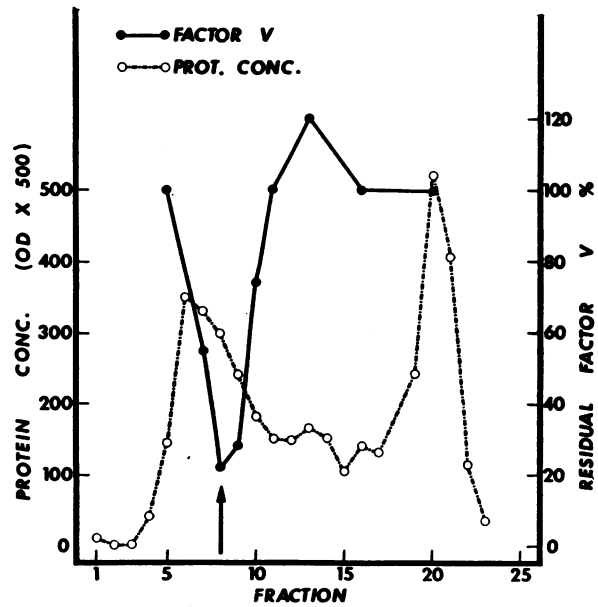

FIGURE 2 Separation of anticoagulant activity from patient No. 1's serum by starch-zone electrophoresis. ½ inch strips were eluted in saline, and the protein concentration of each eluate was determined by a modified Folin method. A portion of each eluate was incubated with an equal volume of normal plasma for 60 min at room temperature, and the mixture was assayed for factor V activity. Protein concentration and factor V activity were then plotted against the electrophoretic location of the eluate. Loss of factor V activity indicated the presence of anticoagulant activity. (Serum from patient No. 2 yielded similar findings.)

Neutralization of anticoagulant activity by immune precipitation. The basic plan for these experiments was to mix the patients' plasmas with different antisera and then to test the supernatant of the mixture for the presence or absence of inhibitor activity. For each experiment to be interpretable two conditions had to be met. (*a*) The patient's plasma had to be diluted in each particular antisera to equivalence or antibody excess. (*b*) Inhibitor activity had to be present when this same dilution of the patient's plasma was made in control normal rabbit sera (nonspecific loss of inhibitor activity in control sera could be due to the following two processes: [*a*] dilution or [*b*] antigenic determinants of factor V in the rabbit sera).

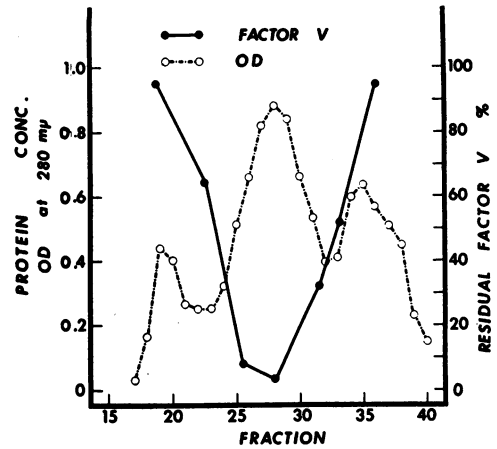

FIGURE 3 Elution of anticoagulant activity after Sephadex G-200 chromatography of patient No. 1's serum. The column was eluted in 4-ml fractions whose protein concentration was determined by absorbancy at 280 mμ. Serial fractions from peaks and valleys were pooled and concentrated. These concentrated fractions were then incubated with normal plasma at room temperature for 60 min, and the mixtures were assayed for factor V activity. Loss of factor V activity indicated the presence of anticoagulant activity. (Serum from patient No. 2 yielded similar findings.)

Because of the lower titer of the inhibitor in patient No. 1, these criteria could not be satisfied in some of the neutralization experiments with patient No. 1's plasma, as indicated in Tables III and IV.

TABLE II

Neutralization of Inhibitor Activity by Rabbit Antihuman Immunoglobulin Antiserum

Supernatant of	Factor V activity of a mixture of the supernatant and a factor V source at	
	0 min	60 min
	%	
Patient No. 1 plasma and normal control rabbit serum	70	7
Patient No. 1 plasma and antihuman immunoglobulin antiserum	70	104
Patient No. 2 plasma and normal control rabbit serum	20	3
Patient No. 2 plasma and antihuman immunoglobulin antiserum	120	120

Sera were diluted in the patients' plasmas in proportions that resulted in equivalence or antibody excess for the antisera. After incubation at 37°C for 1 hr, precipitates were removed by centrifugation. The inhibitor activity of the supernatants was determined by mixing each supernatant with an equal volume of a factor V source (normal plasma) at room temperature and at the times indicated, testing diluted subsamples for factor V activity.

Table II summarizes the results of neutralization experiments using rabbit antihuman immunoglobulin antiserum. As the data illustrate, this procedure removed all evidence of anticoagulant activity in both patients' plasmas and established that the anticoagulants were immunoglobulins.

The experiment was then repeated with antisera specific for γ, α, and μ chains in an attempt to define the immunoglobulin class of the anticoagulants. In patient No. 2 it was possible to show that the anti-γ chain antiserum completely removed the anticoagulant activity from the patient's plasma, whereas the anti-α and anti-μ chain antisera did not remove the anticoagulant activity (Table III). Therefore, the anticoagulant in patient No. 2 was an immunoglobulin of the IgG class.

In patient No. 1, however, it was only possible to show that the anti-μ chain antiserum did not remove the anticoagulant activity (Table IV). It was not possible to determine the effect of the anti-γ or the anti-α chain antisera because the dilution of the patient's plasma in these specific antisera required for equivalence was sufficient in itself to dilute out the inhibitor activity. For example, a 1:10 dilution of the patient's plasma in antiserum was required to remove all of the IgA in the patient's plasma; this same dilution in normal rabbit serum (control) resulted in loss of anticoagulant activity.

TABLE III

Effect of Antisera Against γ, α, and μ Chains upon Inhibitor Activity

Supernatant of	Factor V activity of a mixture of the supernatant and a factor V source at	
	0 min	60 min
	%	
Patient No. 1 plasma and normal rabbit serum (control)	100	24
Patient No. 1 plasma and anti-γ chain serum	n.t.*	n.t.
Patient No. 1 plasma and anti-α chain serum	n.t.	n.t.
Patient No. 1 plasma and anti-μ chain serum	60	9
Patient No. 2 plasma and normal rabbit serum (control)	40	5
Patient No. 2 plasma and anti-γ chain serum	120	120
Patient No. 2 plasma and anti-α chain serum	40	6
Patient No. 2 plasma and anti-μ chain serum	40	5

Test procedure as described in the footnote to Table II.
* n.t. = could not be tested; see text.

A similar problem was encountered in patient No. 1 when we attempted to define the light-chain type of the anticoagulant. It was possible to demonstrate that his antibody contained some λ light-chains (Table IV), but at the dilution of the patient's plasma in anti-κ antiserum required to reach equivalence, anticoagulant activity was also lost from control dilutions in normal rabbit serum.

In patient No. 2, however, light chains could be typed. Antisera specific for either κ or λ chains failed to remove anticoagulant activity from the patient's plasma. Antisera made against Fab fragment, which contains antibodies against both antigenic types of light chains, completely removed the activity (Table IV). Therefore, the inhibitor in patient No. 2 consisted of IgG molecules, some of which contained κ chains and some of which contained λ chains.

Inhibition of heterologous, homologous, and autologous factor V. Since rabbit, dog, and ox plasmas contain much more factor V activity than human plasma, each was diluted with citrated saline to a factor V activity equivalent to that of undiluted human plasma. These diluted plasmas were then incubated with the inhibitors, and subsamples were assayed for factor V activity. Both anticoagulants inhibited factor V activity in all three species to about the same degree as in man.

Conceivably, the patients had an allotype of factor V that differed from the factor V in the blood with which they were transfused. If so, the anticoagulant could be an antibody directed against the transfused factor V and might react more strongly with factor V from other individuals than with the patient's own factor V. This could be tested only with patient No. 1's factor V, since patient No. 2 died while his plasma still contained potent anticoagulant activity which prevented use of his plasma as a source of autologous factor V. Dilutions of patient No. 1's plasma containing his factor V anticoagulant were incubated with dilutions of his plasma after the anticoagulant had disappeared (autologous factor V) and with dilutions of a normal pooled plasma prepared from 10 individuals (homologous factor V). The mix-

TABLE IV

Effect of Antisera Against κ and λ Chains upon Inhibitor Activity

Supernatant of	Factor V activity of a mixture of the supernatant and a factor V source at 0 min	60 min
	%	
Patient No. 1 plasma and normal rabbit serum (control)	76	24
Patient No. 1 plasma and anti-κ chain serum	n.t.*	n.t.
Patient No. 1 plasma and anti-λ chain serum	100	96
Patient No. 2 plasma and normal rabbit serum (control)	32	6
Patient No. 2 plasma and anti-κ chain serum	45	7
Patient No. 2 plasma and anti-λ chain serum	30	5
Patient No. 2 plasma and Fab serum	100	100

Test procedure as described in the footnote to Table II.
* n.t. = could not be tested; see text.

TABLE V

The Effect of the Inhibitor in Patient No. 1 upon Autologous and Homologous Factor V

Incubation time	Residual factor V activity in a mixture of: Patient's recovery plasma and Citrated saline	Patient's original plasma 1:5	Patient's original plasma 1:10	Normal pooled plasma and Citrated saline	Patient's original plasma 1:5	Patient's original plasma 1:10
min						
0	28.0 (82)	37.5 (23)	34.0 (34)	27.0 (98)	39.5 (18)	34.0 (34)
15	28.0 (82)	54.0 (4.3)	36.0 (27)	26.5 (110)	55.5 (3.9)	41.0 (15)
30	29.0 (73)	55.0 (4.0)	36.5 (25)	27.0 (98)	57.0 (3.4)	42.5 (12)
60	29.0 (83)	56.0 (3.6)	37.0 (24)	27.0 (98)	57.5 (3.3)	43.0 (12)

Patient No. 1's recovery plasma (autologous factor V) and a normal pooled plasma from 10 subjects (homologous factor V) were incubated with either citrated saline (control) or patient No. 1's original plasma (factor V inhibitor) diluted 1:5 or 1:10 in citrated saline. At the incubation times listed, subsamples were further diluted 1:5 in citrated saline and assayed for factor V activity. The values are means for three experiments. The numbers outside the parentheses are clotting times in seconds in the factor V assay, and the numbers inside the parentheses are per cent factor V activity.

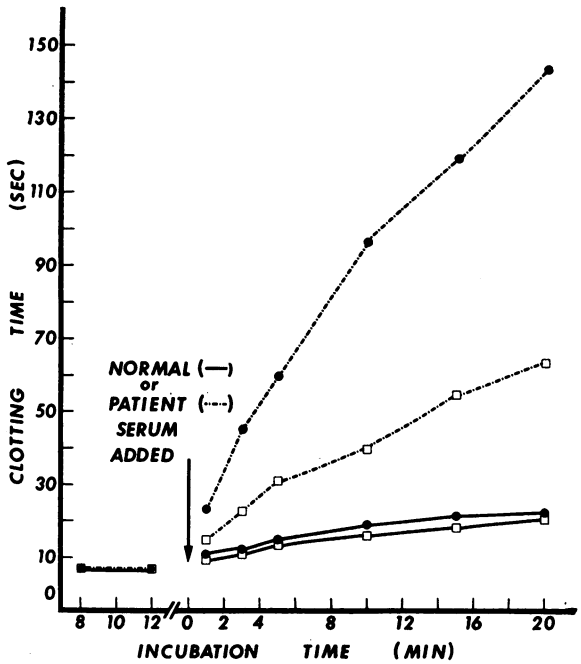

FIGURE 4 Inhibition of intrinsic prothrombin activator by patient No. 1's serum. Identical incubation mixtures were made with equal amounts (0.3 ml) of cephalin suspension, of normal adsorbed plasma 1:5 in barbital buffer, of normal serum 1:10 in barbital buffer, and of 30 mM calcium chloride. At 8 and 12 min, 0.1 ml aliquots were added with 0.1 ml of 20 mM calcium chloride to 0.1 ml of a normal plasma substrate to confirm that prothrombin activator had formed. Then, to one incubation mixture (identified by the solid lines), 0.2 ml of undiluted normal serum was added as a control; to the other incubation mixture (identified by the interrupted lines), 0.2 ml of undiluted patient's serum was added. At the times illustrated, 0.1 ml subsamples were removed from each incubation mixture and added with 0.1 ml of 20 mM calcium chloride to 0.1 ml of hereditary factor V deficiency plasma (identified by solid circles) and to normal plasma (identified by open squares).

tures were then assayed for residual factor V activity. No difference was detectable at a 1:5 dilution of the anticoagulant (Table V). However, at a 1:10 dilution more factor V activity was present after incubation in the patient's recovery plasma than in the pooled normal plasma. Although the difference was small, it was observed each of the three times that the experiment was performed. Thus, the anticoagulant in patient No. 1 appeared to inhibit homologous factor V slightly more effectively than autologous factor V. Unfortunately, we ran out of recovery plasma from patient No. 1 and so could not study the effect of the anticoagulant in patient No. 2 upon patient No. 1's factor V, as compared with homologous factor V in pooled plasma.

Inhibition of platelet factor V activity and plasma factor V activity altered by exposure to thrombin or Russell's viper venom. Platelets contain factor V activity bound to the platelet membrane, which has been shown to be adsorbed plasma factor V (18). The platelets from 20 ml of fresh normal blood were separated by differential centrifugation, washed three times in 30 ml of buffered saline, and resuspended to a final volume of 3 ml in the same fluid. The platelet suspension, 0.5 ml, was then incubated with an equal volume of either hereditary factor V deficiency plasma (control) or patients' plasmas. At the end of 30 min, the platelets which had been incubated with factor V deficiency plasma had a factor V activity of 11%. In contrast, the platelets which had been incubated with either patient No. 1's plasma or patient No. 2's plasma had a factor V activity of 1%. Thus, the anticoagulant inhibited factor V activity bound to platelets.

Dilute thrombin and Russell's viper venom interact with human factor V to increase factor V reactivity as measured in one-stage factor V assays with brain thromboplastin (19). The alterations in the factor V molecule induced by these agents did not prevent loss of factor V activity upon incubation with either anticoagulant. For example, in one experiment one part of 1 u/ml bovine thrombin, prepared as described elsewhere (20), was added to nine parts of oxalated human plasma that had been adsorbed with barium sulfate powder. Clotting times in the factor V assay of diluted subsamples shortened from 45 sec, when barbital buffer was used as the control for the thrombin, to 35–38 sec. An equal volume of either adsorbed herditary factor V deficiency plasma, as a control, or adsorbed patient's plasma was added to the thrombin-treated factor V, and serial subsamples were removed, diluted, and assayed for factor V activity. The clotting times did not lengthen in the subsamples from the mixture containing hereditary factor V deficiency plasma, but they lengthened to 60–75 sec in the subsamples from the mixtures containing each patient's plasma. Similar data were obtained when a 100 μg/ml solution of Russell's viper venom was substituted for the thrombin.

The inhibition of intrinsic prothrombin activator. There was a profound effect of the anticoagulant from both patients upon the generation of intrinsic prothrombin activator in the thromboplastin generation test. As expected, when either patient's blood was the source of the adsorbed plasma, the serum, or both, the incubation mixture failed to generate intrinsic prothrombin activator normally.

Intrinsic prothrombin activator generated from normal reagents clotted normal plasma, hereditary factor V deficiency plasma, and the patients' plasmas in identical times. Since factor V is required for the effective activity of intrinsic prothrombin activator (21–23), these data suggested either that the experimental conditions

had not provided time enough for the anticoagulant in the patients' plasmas to act, or that these anticoagulants could not inhibit factor V after its incorporation into fully formed intrinsic prothrombin activator. Therefore, an experiment was designed in which intrinsic prothrombin activator generated from normal reagents was incubated with the patients' sera or with normal serum (control). Subsamples were removed at increasing intervals and tested for residual prothrombin activator activity by comparing their ability to clot normal plasma and hereditary factor V deficiency plasma. The data for patient No. 1 are illustrated in Fig. 4. Note first that when prothrombin activator was incubated with normal serum, subsamples taken over the next 20 min gave the same clotting times for normal plasma as for hereditary factor V deficiency plasma. Thus, the activity persisting in the mixture was intrinsic prothrombin activator. Intermediates did not contribute significantly to the residual clotting activity. However, when prothrombin activator was incubated with patient No. 1's serum, clotting activity was progressively lost for the normal plasma substrate and, to a striking degree, for the hereditary factor V deficiency plasma substrate. Similar results were obtained with patient No. 2's serum. This experiment clearly established that the inhibitors reacted over time with intrinsic prothrombin activator and confirms that factor V is required for the effective action of the intrinsic prothrombin activator.

Anticoagulant consumption studies. Since both anticoagulants were shown to be immunoglobulins that were consumed in the process of inhibiting factor V clotting activity, we next determined if incubation with factor V antigenic material without clotting activity would also consume the anticoagulants. In preliminary experiments, a dilution of each patient's plasma in normal plasma was found which, after incubation for 30 min, gave complete consumption of the anticoagulant as evidenced by no further inhibition of factor V activity when more normal plasma was added. In subsequent experiments this dilution of the patient's plasma was incubated with material being tested for factor V antigenic determinants. After 30 min, normal plasma was added to supply factor V, and the mixture was tested over time for residual factor V activity.

The data for patient No. 1 are summarized in Fig. 5. Note first that incubation with normal plasma completely consumed the anticoagulant (line *4*), whereas incubation with citrated saline failed to consume the anticoagulant (line *1*). Incubation with human serum, which possessed no factor V clotting activity, also consumed the anticoagulant completely (line *3*) and resulted in clotting times similar to those obtained with plasma. We interpret these data to mean that the anticoagulants had combined with factor V antigenic material without clotting activity and that such antigenic determinants were present in serum.

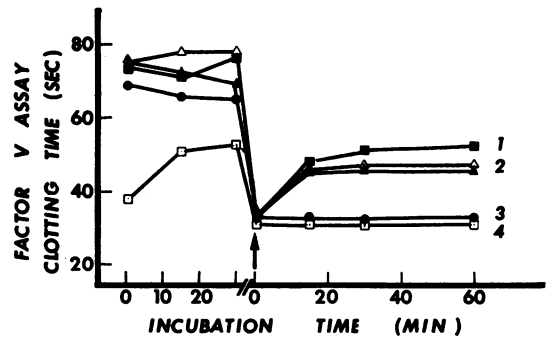

FIGURE 5 Consumption of the factor V anticoagulant in patient No. 1's plasma on incubation with various test materials. To 0.2 ml of patient No. 1's plasma 0.8 ml of the test material was added to give a 1:5 dilution of the factor V inhibitor in the initial incubation mixture. At the times indicated, 0.1 ml subsamples were removed, diluted 1:5 in citrated saline, and assayed for factor V activity. After 30 min incubation at room temperature, an equal volume, 0.7 ml, of normal plasma was added to the incubation mixture to supply factor V. Further subsamples were removed at the times shown, diluted 1:5 in citrated saline, and assayed for factor V activity. (Since the normal plasma was diluted 1:2 on being added to the incubation mixture, the final dilution of test substance in the factor V assay was 1:10.) A citrate-saline blank clotted in about 60 sec in the factor V assay, and a 1:10 dilution of normal plasma clotted in 29 sec. Test materials are indicated as follows: line *1*, citrated saline; line *2*, hereditary factor V deficency plasmas (K.H., M.B.); line *3*, normal serum; line *4*, normal plasma.

Having established that factor V antigenic material without clotting activity would consume the anticoagulant, we then incubated each anticoagulant with plasma from two patients with hereditary factor V deficiency (parahemophilia), one of whom was Owren's original patient (1). Hereditary factor V deficiency plasmas consumed very little anticoagulant activity (line *2*). Identical results were obtained with anticoagulant from patient No. 2. Therefore, the plasma of each of these patients with hereditary factor V deficiency not only lacks factor V activity but also lacks antigenic material of an inactive factor V capable of consuming these anticoagulants.

DISCUSSION

The above data establish that an anticoagulant against factor V was associated with a transient but serious hemorrhagic diathesis in patient No. 1 and serious bleeding in patient No. 2. These anticoagulants appeared to react stoichiometrically with human and other animal factor V in vitro and had the physicochemical characteristics of immunoglobulins. Neutralization of their activity with antihuman immunoglobulin antiserum established that they were both immunoglobulins.

TABLE VI

Factor V Inhibitors Reported in the Literature

Patient No.	Reference	Transfusion prior to development of inhibitor	Duration inhibitor was present	Physicochemical properties
			wk	
1	Ferguson et al. (24)	None	8	Ppt'd with 33–50% $(NH_4)_2SO_4$
2	Lopez et al. (25)	Probable	4	Thought to be immunoglobulin but failed to inactivate bovine factor V
3	Handley et al. (26)	Yes	2	Eluted in first peak on G-200
4	Patient No. 1, this report	Yes	2	See text
5	Patient No. 2, this report	Yes	2	See text

Including our patients, there are now five with factor V inhibitors which have been reported in the English language literature (Table VI). Of particular note is the fact that three, and probably four, of these patients developed the inhibitor within 2 wk of receiving a blood transfusion. Also, in patients Nos. 2, 3, and 4 the inhibitor was transient. Biochemical and immunochemical data on the first three patients were relatively scant. However, the data are not incompatible with the hypothesis that these inhibitors are immunoglobulins. The elution of the inhibitor activity from the first peak on Sephadex G-200 gel filtration in patient No. 3 suggests that this inhibitor was an IgM.

Technical reasons prevented our delineating the immunoglobulin class of the anticoagulant in patient No. 1. However, it was possible to determine that the anticoagulant did not consist of IgM molecules and that at least some molecules contained λ light-chains. Its absence on Sephadex gel filtration from the valley between the first and second peak and strongest activity at the top of the second peak suggests that the anticoagulant did not contain a significant number of IgA molecules. Therefore, we suspect, but cannot prove, that the inhibitor in patient No. 1 belonged to the IgG class.

We were able to establish unequivocably that the anticoagulant in patient No. 2 consisted only of IgG molecules. It was also possible to demonstrate that some of these molecules had κ chains and others had λ chains.

Owren's original studies indicated that factor V participates in prothrombin activation (27), and recent evidence suggests that the prothrombin activator is a complex of activated factor X, factor V, and lipid in the presence of calcium ions (21–23). Ferguson, Johnston, and Howell (24) found that intrinsic prothrombin activator formed from normal reagents gave an abnormal thromboplastin generation test with substrate plasma from their patient with a factor V anticoagulant, which would support this hypothesis. The normal values we found when we did the same experiment were unexpected, and they illustrate a pitfall in test systems for evaluating the site of action of an anticoagulant in which powerful coagulant activity may clot a test mixture before an inhibitor has time to act. Thus, when the experiment was redesigned to allow time for the factor V anticoagulants to react with intrinsic prothrombin activator (Fig. 4), marked inhibition was observed. Our data, therefore, confirm that factor V is required for the effective activity of intrinsic prothrombin activator.

The anticoagulants were immunoglobulins whose activity was consumed not only when they were incubated with plasma containing factor V molecules that had clotting activity, but also when they were incubated with human serum that had no factor V clotting activity (Fig. 5). This experiment established that factor V antigenic material that had lost its clotting activity could still consume inhibitor activity. In contrast, incubation of the anticoagulants with plasma from two patients with hereditary factor V deficiency, one of whom was Owren's original patient (1), failed to consume anticoagulant activity. This indicates that in these patients hereditary factor V deficiency (parahemophilia) represents a true deficiency of factor V molecules rather than the synthesis of a molecule in which a minor structural change results in a loss of clotting activity. This does not eliminate the possibility that other patients with hereditary factor V deficiency may synthesize an abnormal molecule such as has been demonstrated in a minor population of patients with hemophilia A and hemophilia B (28–32).

The reason our patients suddenly started making an immunoglobulin with factor V anticoagulant activity is unknown. Thrombocytopenia, secondary to platelet isoimmunization, is an uncommon but well-documented cause for purpura following transfusions (33, 34). Perhaps the factor V anticoagulants in our patients represent an analogous phenomenon of isoimmunization to a clotting factor after transfusion. In support of this is the fact that four of the five patients with factor V inhibitors received transfusions *prior* to the development

of a bleeding diathesis, and three of five inhibitors were present for a relatively short period of time.

The identification in patient No. 2 of anticoagulant molecules with both light-chain types supports the hypothesis of isoimmunization, because it suggests antibody production by many clones. The rapid disappearance of the anticoagulant in patient No. 1 and its suggestively greater effectiveness against homologous rather than autologous factor V (Table V) also fit with isoimmunization.

These findings contrast strikingly with the accumulating evidence of the monotypic nature of the light-chain types of high titer factor VIII inhibitors, which rarely disappear (35–37).

Bleeding in previously normal individuals because of the formation of an immunoglobulin that inhibits the activity of a hemostatic factor has been described for platelets, for factor VIII, and now for factor V. The transient nature of the anticoagulant in patient No. 1 and our discovery of two cases within 1½ yr lead us to suspect that factor V anticoagulants are not as rare as past documented experience would suggest. As patients with acquired anticoagulants are studied carefully, the evidence grows that the synthesis of immunoglobulins reacting with clotting factors is a major pathogenetic mechanism for acquired hemorrhagic disease in man.

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REFERENCES

1. Owren, P. A. 1947. Parahaemophilia: haemorrhagic diathesis due to absence of a previously unknown clotting factor. *Lancet.* **1**: 446.
2. Proctor, R. R., and S. I. Rapaport. 1961. The partial thromboplastin time with kaolin. A simple screening test for first stage plasma clotting factor deficiencies. *Amer. J. Clin. Pathol.* **36**: 212.
3. Owren, P. A. 1949. A quantitative one-stage method for the assay of prothrombin. *Scand. J. Clin. Lab. Invest.* **1**: 81.
4. Stormorken, H. 1957. The preparation of proaccelerin deficient (parahaemophilia) plasma for the assay of proaccelerin. *Scand. J. Clin. Lab. Invest.* **9**: 273.
5. Hjort, P., S. I. Rapaport, and P. A. Owren. 1955. A simple, specific one-stage prothrombin assay using Russell's viper venom in cephalin suspension. *J. Lab. Clin. Med.* **46**: 89.
6. Rapaport, S. I., and S. B. Ames. 1957. Clotting factor assays on plasma from patients receiving intramuscular or subcutaneous heparin. *Amer. J. Med. Sci.* **234**: 678.
7. Rapaport, S. I., S. B. Ames, and B. J. Duvall. 1960. A plasma coagulation defect in systemic lupus erythematosus arising from hypoprothrombinemia combined with antiprothrombinase activity. *Blood.* **15**: 212.
8. Schiffman, S., S. I. Rapaport, and M. J. Patch. 1965. Starch block electrophoresis of plasma and serum clotting factors. Separation of activated PTC (PTC'). *Blood.* **25**: 724.
9. Rapaport, S. I., P. F. Hjort, M. J. Patch, and M. Jeremic. 1966. Consumption of serum factors and prothrombin during intravascular clotting in rabbits. *Scand. J. Haematol.* **3**: 59.
10. Hougie, C. 1962. A simple assay method for factor X (Stuart-Prower Factor). *Proc. Soc. Exp. Biol. Med.* **109**: 754.
11. Rapaport, S. I., S. Schiffman, M. J. Patch, and A. G. Ware. 1961. A simple, specific one-stage assay for plasma thromboplastin antecedent activity. *J. Lab. Clin. Med.* **57**: 771.
12. Schiffman, S., S. I. Rapaport, and M. J. Patch. 1963. The identification and synthesis of activated plasma thromboplastin component (PTC'). *Blood.* **22**: 733.
13. Kunkel, H. G. 1954. Zone electrophoresis. *Methods Biochem. Anal.* **1**: 141.
14. Fahey, J. L., and A. P. Horbett. 1959. Human gamma globulin fractionation on anion exchange cellulose columns. *J. Biol. Chem.* **234**: 2645.
15. Flodin, P., and J. Killander. 1962. Fractionation of human-serum proteins by gel filtration. *Biochim. Biophys. Acta.* **63**: 403.
16. Franklin, E. C. 1960. Structural units of human 7S γ globulin. *J. Clin. Invest.* **39**: 1933.
17. Kabat, E. A., and M. M. Mayer. 1964. Experimental Immunochemistry. Charles C. Thomas, Springfield, Ill. 2nd edition. 905.
18. Hjort, P., S. I. Rapaport, and P. A. Owren. 1955. Evidence that platelet accelerator (platelet factor 1) is adsorbed plasma proaccelerin. *Blood.* **10**: 1139.
19. Rapaport, S. I., S. Schiffman, M. J. Patch. 1966. Rabbit factor V: different effects of thrombin and venom, a source of error in assay. *Amer. J. Physiol.* **211**: 1477.
20. Rapaport, S. I., S. Schiffman, M. J. Patch, and S. B. Ames. 1963. The importance of activation of antihemophilic globulin and proaccelerin by traces of thrombin in the generation of intrinsic prothrombinase activity. *Blood.* **21**: 221.
21. Papahadjopoulos, D., and D. J. Hanahan. 1964. Observations on the interaction of phospholipids and certain clotting factors in prothrombin activator formation. *Biochim. Biophys. Acta.* **90**: 436.
22. Cole, R. R., J. L. Koppel, and J. H. Olwin. 1965. Phospholipid-protein interactions in the formation of prothrombin activator. *Thromb. Diath. Haemorrh.* **14**: 431.
23. Jobin, F., and M. P. Esnouf. 1967. Studies on the formation of the prothrombin-converting complex. *Biochem. J.* **102**: 666.
24. Ferguson, J. H., C. L. Johnston, Jr., and D. A. Howell. 1958. A circulating inhibitor (Anti-AcG) specific for the labile factor V of the blood-clotting mechanism. *Blood.* **13**: 382.
25. Lopez, V., R. Pflugshaupt, and R. Butler. 1968. A specific inhibitor of human clotting factor V. *Acta Haematol.* **40**: 275.
26. Handley, D. A., and B. M. Duncan. 1969. A circulating anticoagulant specific for factor V. *Pathology.* **1**: 265.
27. Owren, P. A. 1947. The coagulation of blood. Investigations on a new clotting factor. *Acta Med. Scand.* **128** (Suppl.): 194.
28. Hougie, C., and J. J. Twomey. 1967. Hemophilia B_m: a new type of factor IX deficiency. *Lancet.* **1**: 698.

29. Roberts, J. R., J. E. Grizzle, W. D. McClester, and G. D. Penick. 1968. Genetic variants of hemophilia B: detection by means of a specific inhibitor. *J. Clin. Invest.* **47**: 360.
30. Hoyer, L. W., and R. T. Breckenridge. 1968. Immunologic studies of antihemophilic factor: cross reacting material in a genetic variant of hemophilia A. *Blood J. Hematol.* **32**: 962.
31. Feinstein, D. I., M. M. Y. Chong, C. Kasper, and S. I. Rapaport. 1969. Hemophilia A: polymorphism detectable by a factor VIII antibody. *Science (Washington).* **163**: 1071.
32. Denson, K. W. E. 1968. Two forms of haemophilia. *Lancet.* **2**: 222.
33. Shulman, N. R., R. H. Aster, A. Leitner, and M. C. Hiller. 1964. Platelet and leukocyte isoantigens and their antibodies: serologic, physiologic, and clinical studies. *In* Progress in Hematology. C. V. Moore and E. Brown, editors. Grune & Stratton, Inc., New York. **4**: 22.
34. Morrison, F. S., and P. L. Mollison. 1966. Post-transfusion purpura. *N. Engl. J. Med.* **275**: 243.
35. Shapiro, S. S. 1967. The immunologic character of acquired inhibitors of antihemophilic globulin (factor VIII) and the kinetics of their interaction with factor VIII. *J. Clin. Invest.* **41**: 147.
36. Shapiro, S. S., and K. S. Carroll. 1968. Acquired factor VIII antibodies: further immunologic and electrophoretic studies. *Science (Washington).* **160**: 786.
37. Feinstein, D. I., S. I. Rapaport, and M. M. Y. Chong. 1969. Immunologic characterization of 12 factor VIII inhibitors. *Blood. J. Hematol.* **34**: 85.