Heterogeneity of Human Factor V Deficiency

EVIDENCE FOR THE EXISTENCE OF ANTIGEN-POSITIVE VARIANTS

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ABSTRACT Functional human Factor V has been purified using a rapid immunoaffinity method. Following barium citrate adsorption of plasma, Factor V was precipitated with polyethylene glycol at a concentration between 5 and 14%. The resulting preparation was applied to a column containing an immobilized immunoadsorbent consisting of an IgG fraction containing a naturally occurring human monoclonal (IgG_4 λ) antibody with inhibitory activity against human Factor V. The solid phase immunoglobulin quantitatively bound Factor V from human plasma. The bound Factor V was effectively eluted with a Tris buffer pH 7.2 containing 1.2 M NaCl and 1 M α-methyl-D-mannoside. The isolated native Factor V with high specific activity (92 U/mg) showed a single band (M_r, 350,000) on both reduced and nonreduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Factor V was purified 5,100-fold over plasma with an overall yield of 77%. The purified Factor V when subjected to thrombin activation exhibited an 18-fold increase in coagulant activity.

The isolated Factor V neutralized the inhibitory activities of the monoclonal antibody that was used to purify it, as well as the rabbit antibodies produced by immunizing the animals with the purified Factor V. Immunoelectrophoresis of purified Factor V against the polyclonal rabbit antiserum resulted in a single precipitin arc of identical mobility to the Factor V in normal human plasma. Analysis by double immunodiffusion showed a line of identity between plasma and purified Factor V and crossed immunoelectrophoresis showed a single species in normal plasma.

A competitive enzyme-linked immunosorbent assay using the rabbit antibody against Factor V was applied to quantify Factor V antigen level in human plasma. Reconstitution of congenitally deficient or immunodepleted plasma with normal plasma or purified Factor V gave parallel dose-response curves. In 14 normal plasma the coagulant activity was 0.98±0.02 U/ml (mean±SEM) and antigen concentration was 11.1±0.4 μg/ml. A pool of 14 patients with congenital Factor V deficiency were studied. 10 patients had Factor V antigen ranging from 1.0 to 2.4 μg/ml with corresponding coagulant activities (0–0.17 U/ml) indicating a low concentration of normal Factor V, presumably due to decreased synthesis or increased degradation. When these patient plasmas and the normal plasmas were analyzed together an excellent correlation ($r = 0.97$, $P < 0.01$) was obtained. However, four patients with coagulant activity (0–0.08 U/ml) had Factor V antigen concentrations ranging from 4.4 to 6.1 μg/ml, indicating the presence of a reduced concentration of abnormal Factor V protein. The presence of patients with antigen similar in concentration to coagulant activity and antigen in excess of Factor V activity indicates the heterogeneity of congenital Factor V deficiency.

INTRODUCTION

Factor V, a plasma coagulation cofactor, is necessary for the optimal rate of conversion of prothrombin to thrombin catalyzed by the serine protease Factor Xa in the presence of calcium and phospholipid (1, 2). Because of its sensitivity to proteolysis and thermal denaturation, human Factor V has been difficult to isolate in high yield. Human Factor V has been purified to apparent homogeneity by conventional techniques (3, 4) and by the use of a mouse monoclonal

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antibody to human Factor V (5). The former techniques gave yields of 6 and 21%, respectively, while the latter gave a yield of 28%.

Hurtubise et al. (6) isolated an immunoglobulin from the serum of a patient with a fatal hemorrhagic diathesis, which had the characteristics of a monoclonal antibody against Factor V. The protein was an IgG_4 (λ) with restricted mobility on immunoelectrophoresis, which had neutralizing activity against human plasma Factor V (6). We have used this immunoglobulin in the present study to develop a high-yield purification of human Factor V.

Heterologous antibodies were produced against immunoaffinity purified Factor V, which precipitate Factor V in human plasma and neutralize its coagulant activity. This antiserum was used to develop a competitive enzyme-linked immunosorbent assay (ELISA)[1] in order to investigate whether the genetic defect in some patients is due to the synthesis of abnormal molecules lacking procoagulant activity or to decreased synthesis of an apparently normal molecule. We have identified four individuals with a variant form of Factor V molecule, which is antigenically reactive with a polyclonal rabbit antibody, but not with a naturally occurring human antibody.

METHODS

Reagents. Benzamidine hydrochloride, soybean trypsin inhibitor, phenylmethyl sulfonyl fluoride, diisopropylfluorophosphate, aprotinin, *p*-nitrophenyl phosphate and goat anti-rabbit IgG conjugated with alkaline phosphatase were supplied by Sigma Chemical Co., St. Louis, MO. Polyethylene glycol 6000 (PEG) was obtained from Fisher Scientific Co., Pittsburgh, PA. Reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA. Purified human thrombin (2,900 U/mg) was a gift from Dr. John Fenton III, NY State Department of Health, Albany, NY. DE-52 cellulose was obtained from Whatman, Inc., Clifton, NJ. Protein A coupled to Sepharose CL-4B and cyanogen bromide-activated Sepharose 4B from Pharmacia Fine Chemicals, Piscataway, NJ. All the chemicals used were reagent grade or better.

Source of normal and patient plasma samples. Blood was drawn from normal volunteers by venepuncture into plastic bottles containing 0.13 M trisodium citrate (9:1 vol/vol). Platelet-free plasma was obtained by centrifugation of the anticoagulated blood at 2,000 *g* for 15 min at 4°C. Frozen plasma from 10 different patients congenitally deficient in Factor V was kindly provided by Drs. Carol Kasper, Los Angeles, CA, Frederick Dombrose, Chapel Hill, NC, Douglas Triplett, Indianapolis, IN, Sandra Schiffman, Los Angeles, CA, Helen I. Glueck, Cincinnati, OH, and Oscar Ratnoff, Cleveland, OH. One of Dr. Triplett's patients with congenital deficiency had acquired an antibody secondary to transfusions. Three additional Factor V-deficient plasmas as well as normal pooled plasma (20 donors) were purchased from George King, Biomedical, Inc., Overland Park, KS. One individual plasma obtained from Dr. Ratnoff had combined Factor V (0.17 U/ml) and Factor VIII (0.16 U/ml) deficiency.

For certain experiments Factor V in plasma was inactivated by incubation at 56°C for 30 min or with EDTA 10 mM pH 7.5, at 37°C for 5 h. Such Factor V had <0.01 U/ml activity by coagulant assay.

Plasma for purification. 1,500 ml of blood was drawn and processed as described above except that the anticoagulant contained added protease inhibitors at final concentrations of 1 mM benzamidine hydrochloride, 5.5 mM diisopropylfluorophosphate, 1.0 mM phenylmethylsulfonylfluoride, 2.4 μM soybean trypsin inhibitor, and 100 KIU/ml aprotinin. After centrifugation the fresh plasma was then processed to isolate the Factor V.

Purification of a human immunoglobulin with Factor V inhibitory activity. The IgG_4 (λ) antibody was isolated from a patient's plasma (6), which contained inhibitory activity against human Factor V. A γ-globulin-containing fraction was precipitated from the antiserum by 33% saturated ammonium sulfate. The precipitate was redissolved and dialyzed against 0.01 M phosphate buffer, pH 8.0 to remove the ammonium sulfate and was further chromatographed on DE-52 cellulose using a linear gradient from 0.01 M to 0.3 M phosphate, pH 8.0. The first protein peak eluting at ~0.02 M phosphate, capable of neutralizing the Factor V-clotting activity at a dilution of >1:100, was subjected to further purification by affinity chromatography on protein A-Sepharose CL-4B. The IgG was eluted from the affinity column with 1 M acetic acid and neutralized immediately by collecting into tubes containing 4 vol of 0.2 M $NaHCO_3$ buffer pH 8.3 in 0.4 M NaCl.

Preparation of the immobilized immunoadsorbent. The purified IgG containing the antibody was dialyzed against 0.1 M $NaHCO_3$ buffer, pH 8.3 before coupling to the washed cyanogen bromide-activated Sepharose 4B (7). The immunoadsorbent was then equilibrated in 0.02 M Tris buffer pH 7.2 containing 0.15 M NaCl and 0.02% NaN_3. The coupling efficiencies under the conditions used in this study ranged from 70 to 95% and the final density of the IgG antibody coupled on the gel was 1–3 mg/ml of gel.

Factor V-immunodeficient plasma. The plasma from a normal volunteer was rendered deficient in both Factor V activity and antigen by passage through the immobilized immunoabsorbent of human antibody to Factor V previously described. The procedure results in <0.001 U/ml detectable Factor V remaining in the plasma by coagulant assay and <10 ng/ml by ELISA (see below).

Production of rabbit antiserum against Factor V. Purified human Factor V (see below) was used to immunize rabbits to produce the polyclonal anti-Factor V antibodies. For each rabbit 200 μg of purified Factor V homogenized with equal volume of complete Freund's adjuvant was injected subcutaneously. 2 wk later, the rabbits were boosted with the same amount of Factor V in an equal volume of incomplete Freund's adjuvant and blood collected on days 8, 10, and 12 after each booster injection. To remove small amounts of other antibodies the rabbit antiserum was adsorbed by incubating the serum with 1:10 vol of Factor V-immunodeficient plasma at 37°C for 1 h followed by 18 h at 4°C. The immunoprecipitate was removed by centrifugation. The process was repeated three to five times until no precipitate was observed. The coagulant activity of rabbit Factor V in the antiserum was inactivated by incubation at 58°C for 30 min.

[1] *Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay; PEG, polyethylene glycol 6000.

Coagulant assay of Factor V. The assay of Factor V-clotting activity was performed by the one-stage method of Ware et al. (8) using congenitally or artificially deficient plasma. 1 U of Factor V is defined as the amount in 1 ml of normal human plasma.

Determination of Factor V antigen levels by ELISA. A competitive ELIA (9) was used to quantify the Factor V antigen levels using the rabbit polyclonal antibodies against Factor V and the purified Factor V. In the assay, purified Factor V was diluted with 0.02 M Tris buffer, 0.15 M NaCl, pH 7.4 to a final concentration of 0.5 μg/ml, based on the specific activity of purified Factor V of 92 U/mg (see Results). The Factor V was coated onto the surface of polystyrene cuvettes by incubating 0.2 ml of the diluted solution containing 100 ng of Factor V in wells of the polystyrene cuvettes for 18 h at 4°C. The excess unadsorbed protein was then removed by extensive washing with 0.02 M Tris buffer, pH 7.4. 0.1 ml of antiserum (1:640) diluted in 0.02 M Tris pH 7.4 containing 0.15 NaCl and 0.05% Tween 20 (assay buffer) was found to contain just enough antibody to neutralize 0.01 U of Factor V coagulant activity. This amount of antiserum was then incubated with 0.1 ml of various dilutions of Factor V-deficient plasma in the wells coated with purified Factor V. At the same time a standard curve was obtained by incubating same amount of rabbit antiserum with equal volumes of dilutions of pooled normal human plasma in similar wells. A control of similar diluted pooled normal human plasma was incubated with rabbit preimmune normal serum and used to correct for nonspecific binding. All dilutions were made with the assay buffer. After incubation for 3 h at 25°C the unbound protein was removed by extensive washing with assay buffer. An excess amount of the IgG fraction immunoglobulin of goat anti-rabbit IgG antiserum conjugated with alkaline phosphatase, was incubated in the wells for 3 h at 25°C. Excess unreacted antibody-enzyme conjugate was washed away. The amount of antibody-enzyme conjugate that was bound to the rabbit anti-Factor V antibody complexed with Factor V to the solid phase was determined by its action on a chromogenic substrate, *p*-nitrophenyl phosphate. 0.2 ml of the chromogenic substrate solution (1 mg/ml) in 0.1 M glycine buffer, pH 10.4 containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$, was incubated in the cuvettes at 25°C and at suitable time the reaction was stopped by adding 0.2 ml of 1 N NaOH. The color developed was read at 405 nm in a spectrophotometer. The control consisting of rabbit antiserum incubated in the absence of any source of human Factor V was taken as 100% of the antibody bound. The absorbance of the control with preimmune rabbit serum alone instead of antiserum was subtracted to correct for nonspecific binding. In general, <10% of the measured absorbance was due to nonspecific binding. All samples were assayed in triplicate and a linear standard curve was obtained by plotting the average of the absorbances of the triplicates vs. log dilution of the pooled normal plasma. Each Factor V-deficient plasma was assayed at three different dilutions (undiluted, 1:2, 1:10) each in triplicate. The average absorbance for the dilutions, which fell in the linear range of the standard curve, was used to calculate the Factor V antigen levels.

In addition to the normal plasma standard curve the validity of the assay was tested by adding known amounts of purified Factor V or normal plasma to plasma rendered deficient in Factor V by immunoabsorption or to congenitally Factor V-deficient plasma with Factor V antigen < 10 ng/ml by ELISA.

Immunochemical procedures. Double immunodiffusion (10) was performed on 1% agarose gel in phosphate buffer, pH 7.4 using rabbit anti-Factor V antiserum. Immunoelectrophoresis (10) was performed on 1% agarose gel in barbital buffer, pH 8.6 ionic strength (μ) = 0.02 at 16 mA/slide. Crossed immunoelectrophoresis (10) was performed on 1% agarose gel in barbital buffer pH 8.6. Plasma was electrophoresed for 2 h at 200 V at 23°C. The gel was then cut and applied to one end of a second glass plate and the remaining space filled with 1% agarose gel containing 1% rabbit anti-Factor V antiserum and the gel electrophoresed at 50 V for 18 h at 15°C perpendicular to the initial electrophoresis. The precipitin arcs in all three procedures was then stained with Coomassie Blue, after extensive washing the plates with 0.1 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.2.

The activity of the antibodies against purified human Factor V was monitored by their capacity to neutralize the Factor V-clotting activity. The titer of the antibodies in each antiserum was compared by determining the dilutions of the heat-inactivated antisera at which 50% of Factor V-clotting activity in normal plasma will be neutralized. The procedure of the neutralization assay was as follows: dilutions of antiserum were incubated with equal volume of pooled normal plasma or purified Factor V solution containing 1 U activity/ml overnight (18 h) at 4°C. The residual Factor V activity after neutralization was determined from the observed clotting times of each sample by reference to a standard curve for the coagulant assay.

Thrombin activation of Factor V. Activation of purified Factor V was performed by incubating purified Factor V with two concentrations of thrombin at 24°C and the final volume adjusted to 600 μl with coagulant assay buffer. Aliquots of the mixture were taken at specific times for assay. The coagulant activity of Factor V before thrombin activation was also measured and used in the calculation of activity enhancement of activated Factor V. The extent of activation was expressed as the ratio of activities before and after thrombin activation.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed using a Tris buffer system (11). Three different gel concentrations, 3, 4, and 5%, and both reduced (dithiothreitol 1 mM) and nonreduced samples were electrophoresed. Protein bands were stained with Coomassie Brilliant Blue R. Molecular weights of unknowns were calibrated from the standard curves constructed with the following molecular weight standards: myosin (200,000), β-galactosidase (116,000), phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), and purified fibrinogen (330,000), under nonreducing conditions.

RESULTS

Purification of human Factor V. Isolation of human Factor V from plasma in a native state has been difficult because of the labile nature of this specific coagulant factor and its extreme sensitivity to proteolytic enzymes. The purification steps were rapid and performed without interruption to minimize the time of manipulation, which can be as little as 8 h. Protease inhibitors were included during blood collection and throughout the purification procedures. $BaCl_2$ was added dropwise to the anticoagulated plasma until a final concentration of 80 mM was reached and the mixture was stirred for 1 h at 4°C. The barium citrate precipitate was then removed by centrifugation at

10,000 *g* for 20 min. The Factor V activity was quantitatively recovered in the supernatant.

Presumably both prothrombin and protein C, precursors of enzymes to which Factor V is most susceptible, were removed rapidly by barium citrate adsorption without loss of activity (Table I). Solid PEG was then added to a final concentration of 5% and stirred for 1 h at 4°C. The precipitate, free of Factor V activity, was removed by centrifugation at 10,000 *g* for 20 min. Additional PEG was then added to the supernatant to a final concentration of 14% and stirred for another 1 h. The supernatant that was almost free of Factor V activity was removed after centrifugation at 10,000 *g* for 20 min. The PEG precipitation, which gave a 2.8-fold purification with an almost quantitative yield, also allows concentration of activity for the final step. The 14% PEG precipitate was then redissolved in 0.02 Tris buffer, 0.15 M NaCl pH 7.4 and was applied to a 70-ml immunoadsorbent column, when no >20 U of Factor V were applied for each millilter immunoadsorbent (1–3 mg IgG), >95% of the Factor V activity was bound. 10-ml plastic columns containing immunoadsorbent were washed thoroughly with 0.02 M Tris buffer, pH 7.4 containing 0.15 M NaCl, 0.02% NaN_3 before use. Factor V was passed through the column in the same buffer at a flow rate of 20 ml/h, or more efficiently, was recycled several times through the column at the same flow rate until negligible amount of Factor V activity was detected in the effluent. When Sepharose 4B without coupled specific IgG was used, little Factor V bound to the gel. To eliminate any proteins that were bound either through ionic or hydrophobic interactions, the column was washed with buffers containing, respectively, high salt and high sugar concentrations before elution of the bound Factor V. 20 vol each of 0.02 M Tris buffer, pH 7.4 containing 0.65 M NaCl and 0.02 M Tris buffer, pH 7.4 containing 1 M α-methyl-D-mannoside eliminated nonspecifically bound molecules.

The bound Factor V was not eluted by either buffer when the densities of the IgG on the immunoadsorbents were >2 mg/ml of gel. Bound Factor V was then eluted at 25°C from the immunoaffinity gel with 0.2 M Tris buffer containing 5 mM $CaCl_2$, 1.2 M NaCl, and 1 M α-methyl-D-mannoside. The purified Factor V was then dialyzed against 0.02 M Tris buffer containing 5 mM $CaCl_2$ pH 7.4 and concentrated by ultrafiltration. The concentrated Factor V solution was then stored at −70°C in 25% glycerol. The results of the three-step isolation procedure are summarized in Table I. The final product of Factor V from this three-step procedure was 5,100-fold purified with an overall yield of 77% from the original plasma. Virtually all the purification occurred at the immunoaffinity step.

Characterization of the immunoaffinity-purified Factor V. SDS-polyacrylamide gel electrophoresis (Fig. 1) of the reduced and nonreduced purified Factor V showed a single band at position corresponding to a molecular weight of 350,000. No major differences were noted in molecular weight using 3, 4, and 5% gels (data not shown). This purified Factor V (3.8 U/ml) can be activated by thrombin (0.1 U/ml) with associated 18-fold increase of Factor V-clotting activity at 24°C, equivalent to that previously reported from this laboratory using diluted human plasma (12). The cleavage pattern on 5% SDS gels (data not shown) was similar to that previously reported (3–5, 13). The concentration of thrombin used did not affect the coagulant assay. The activation and gel data indicate that the purified Factor V is a homogenous single chain comparable to previously reported preparations (3–5, 13). When stored in a concentration of 0.25 mg/ml or above at −80°C in 25% glycerol in Tris buffer, pH 7.4 no significant loss of Factor V activity was observed at 2 mo. Purified Factor V can neutralize the inhibitory activities of human monoclonal anti-human Factor V antibody (Fig. 2) as well as the rabbit antibody (data not shown).

Specificity of rabbit anti-human Factor V antibody. The antibody initially produced one major and two barely visible precipitin lines against normal plasma by double immunodiffusion, however, only one pre-

TABLE I
Purification of Human Factor V

Step	Volume	Protein	Factor V activity	Yield	Specific activity	Purification
	ml	*mg*	*U*	*%*	*U/mg*	*-fold*
Citrated plasma	1500	84,900	1,500	100	0.018	1.0
Ba citrate	1620	78,730	1,520	101	0.020	1.1
PEG (5–14%)	1000	35,800	1,350	90	0.038	2.8
Immunoaffinity chromatography	50	12.5	1,150	77	92.0	5,100

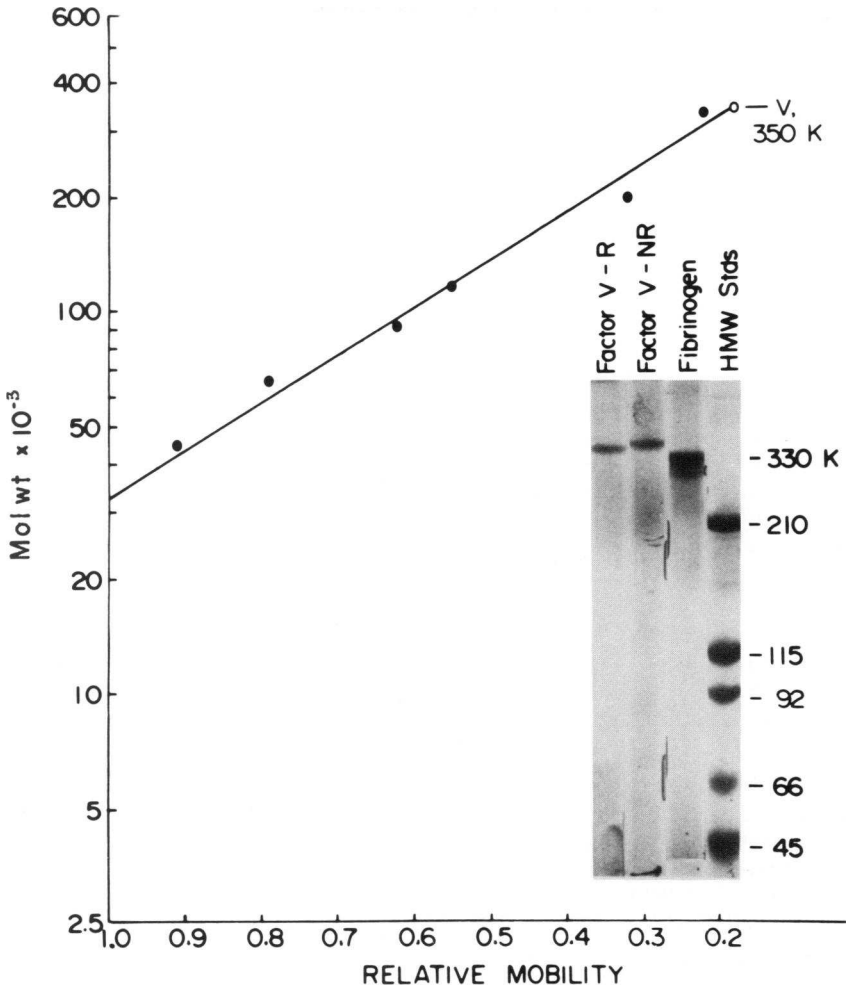

FIGURE 1 SDS-polyacrylamide gel electrophoresis of purified Factor V. The molecular weight standards included are purified fibrinogen (330,000), myosin (200,000), β-galactosidase (115,000), phosphorylase B (92,000), bovine serum albumin (66,000), and ovalbumin (45,000). A calibration curve of log M_r vs. distance migrated in the gel is plotted. Protein bands were stained with Coomassie Brilliant Blue R. Both reduced (R) and nonreduced (NR) purified Factor V are included.

cipitin line was found against the purified Factor V. The antiserum was therefore rendered monospecific by adsorptions with plasma rendered free of Factor V by immunoabsorption. The adsorbed rabbit antisera then showed single precipitin lines against normal plasma and purified Factor V with a reaction of identity (Fig. 3 *a*). The convex shape and position of the precipitin lines near the antigen well reflects the higher molecular weight of antigen relative to that of the antibody. Immunoelectrophoresis of the adsorbed antibodies against the purified Factor V and normal plasma also showed single precipitin lines with identical electrophoretic mobility in the α_2-globulin position for both (Fig. 3 *b*). The adsorbed rabbit antiserum was shown to be monospecific by crossed immunoelectrophoresis, since a single precipitin arc was observed against concentrated pooled normal plasma (Fig. 4, *top*). No precipitin arc was observed against Factor V-deficient plasma at the same position (Fig. 4, *bottom*) indicating that the polyclonal rabbit anti-Factor V antiserum was monospecific against Factor V. The rabbit antiserum diluted 1:64 could neutralize >90% of Factor V-coagulant activity in an equal volume of purified Factor V (1 U/ml) or pooled normal plasma (Methods), while preimmune serum had no such effect.

Assay of Factor V in plasma from normal and Factor V-deficient individuals. A representative concen-

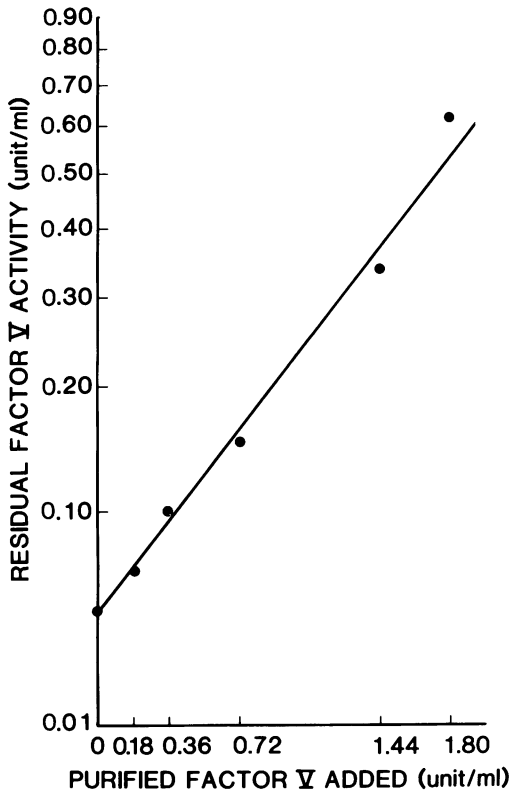

FIGURE 2 Neutralization of anti-human Factor V inhibitory activity by purified Factor V. Various concentrations of purified Factor V were mixed with an equal volume of a human antiserum dilution that was enough to neutralize 96% of Factor V activity (1 U/ml) in normal plasma and incubated for 2 h at 25°C. 2 vol of the above mixture were then incubated with 1 vol of pooled normal plasma for 18 h at 4°C. The residual Factor V activity as then calculated from the measured clotting time of the above mixtures.

tration curve (Fig. 5) of the ELISA is displayed in which the absorption of the chromogenic substrate hydrolyzed by the alkaline phosphate conjugated to the second antibody (goat anti-rabbit IgG) is plotted against the Factor V in normal pooled plasma or purified Factor V added in the assay. In preliminary studies, the absorbance was found to be proportional to the amount of first antibody bound to the plate. Since this is a competition assay the log of the activity of the conjugated indicator enzyme is inversely proportional to the log of the amount of Factor V added in the form of pooled normal plasma. Using such a plot, the assay has a linear range of 7.5–240 ng of plasma Factor V antigen added. Since the concentration in normal pooled plasma is defined as 1 U/ml and the specific activity of purified Factor V is 92 U/mg (Table I) the antigen concentration in the pooled plasma is 10.9 μg/ml. The validity of the assay rests on the specificity of the antiserum, the purity of the Factor V used for coating the plates and the accuracy of the assay. Plasma immunodepleted of Factor V was reconstituted with either pooled normal plasma or purified Factor V (Fig. 6). The lines were virtually parallel (slopes of −0.267 and −0.258, respectively). Thus, the antiserum detected purified Factor V and plasma Factor V similarly in a plasma milieu. These results indicate that this antiserum can accurately measure Factor V in plasma, since the recovery of either added normal plasma or purified Factor V was similar. There also appears to be no detectable cross-reaction with another plasma protein antigen. To further support these conclusions we reconstituted plasma from a patient congenitally deficient in Factor V (<0.01 μg/ml) with normal pooled plasma or purified Factor V. As assessed by ELISA, the lines were again parallel (slope of −0.257 and −0.260, respectively). Since the slopes of the lines based on a reconstitution of Factor V-immunodepleted plasma and plasma from a patient congenitally deficient in Factor V were almost identical, the specificity of the assay is validated. With 12 replicates of the same sample the interassay coefficient of variation was ±10%.

We then applied the ELISA to 14 normal individuals as well as 14 patients with Factor V deficiency as judged by the coagulant assay (Fig. 7). In 14 normal individuals Factor V coagulant activity was 0.98±0.04 U/ml (mean±SEM) while the mean of the antigen level was 11.1±0.4 μg/ml, similar to the value calculated for normal pooled plasma 10.9 μg/ml. The normal specific activity was 88.2 U/mg, closely in agreement with the specific activity of purified Factor V. In 10 of the patients with congenital deficiencies the coagulant activity (0–0.17 U/ml) was congruent with the antigen levels (1.0–2.4 μg/ml). The average specific activity of these deficient individuals was 47 U/mg. When these 24 individuals were analyzed together by linear regression, there was an excellent correlation ($r = 0.97$, $P < 0.01$) between the coagulant activity and antigen concentration. These included both the patient with congenital Factor V deficiency and an acquired antibody to Factor V and the individual with combined Factor V and Factor VIII deficiency. However, this correlation did not include four patients with Factor V antigen concentrations (4.4–6.1 μg/ml), considerably in excess of coagulant activity (0–0.07 U/ml). The average specific activity of the four variant patients was 9.2 U/mg, ~10% of the normal specific activity. This disparity between activity and antigen indicated that nonfunctional Factor V protein was present. This situation could be mimicked by inactivation of Factor V in normal plasma by exposure to high temperature or EDTA. The coagulant activity was no longer detectable but the antigen concentration was unaltered.

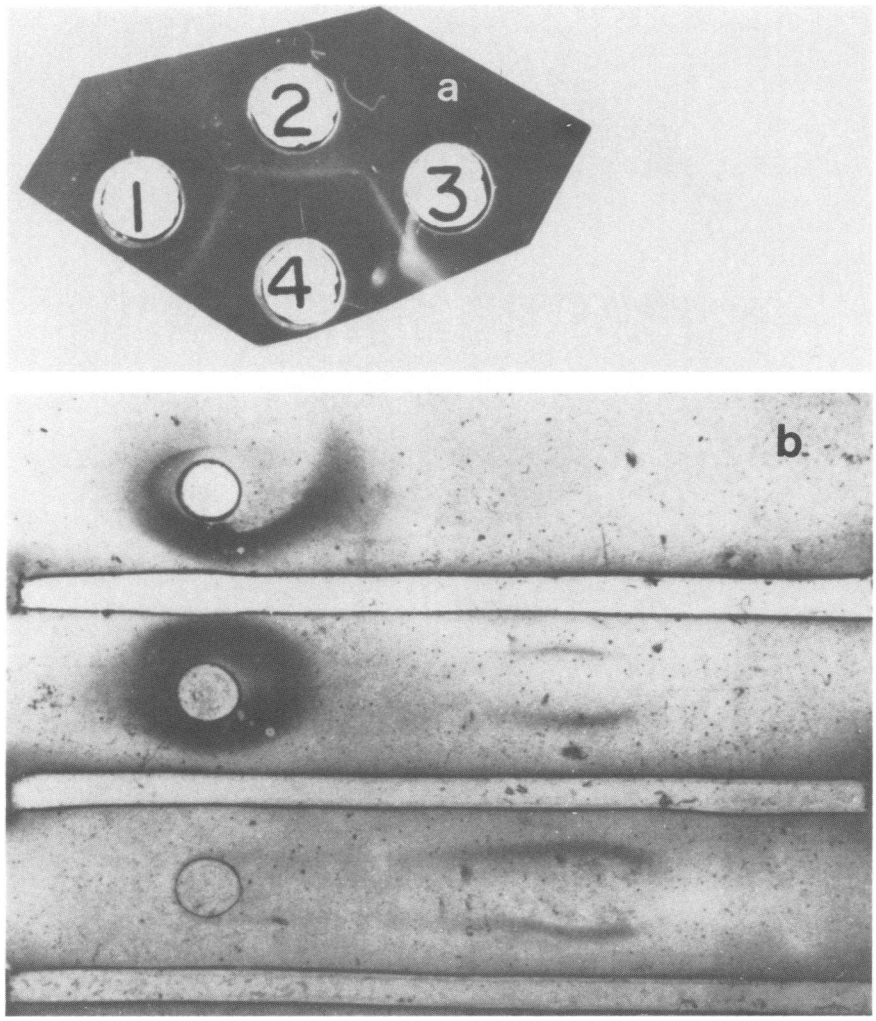

FIGURE 3 (*a*) Double immunodiffusion of Factor V. Polyclonal rabbit anti-human Factor V placed in well 4 diffused against two aliquots of purified Factor V in wells 1 and 3 and a pooled normal plasma in well 2. (*b*) Immunoelectrophoresis of Factor V. Immunoelectrophoresis was performed in a 1% agarose gel in barbital buffer, pH 8.6 (Methods). The upper well contains a Factor V-deficient plasma, the middle well normal plasma, and the lower well purified Factor V. After electrophoresis the antigen was diffused against a polyclonal rabbit anti-Factor V antiserum in troughs.

DISCUSSION

Our human Factor V preparation appears as a single component M_r = 350,000 on both reduced and nonreduced gels. Dahlbäck (3) purified human Factor V with an overall 6% yield. When that material was gel filtered on Ultrogel 22 the largest component had a M_r of 330,000, but smaller components containing 75% of the Factor V activity with higher specific activity representing activated Factor V were also isolated. Suzuki et al. (13) improved their procedure by including proteolytic inhibitors and substantially improved the yield to 24%. In our preparation, in the presence of protease inhibitors, proteolytic fragments are not usually detected by SDS gel electrophoresis. Moreover, fragments of Factor V were not detected on immunoelectrophoresis. Kane and Majerus (4) purified human Factor V to apparent homogeneity with an M_r of 335,000 with a 21% yield. We have repeated their first two steps precipitation with PEG followed by barium citrate adsorption and obtained a similar preparation of Factor V 17-fold purified. Application

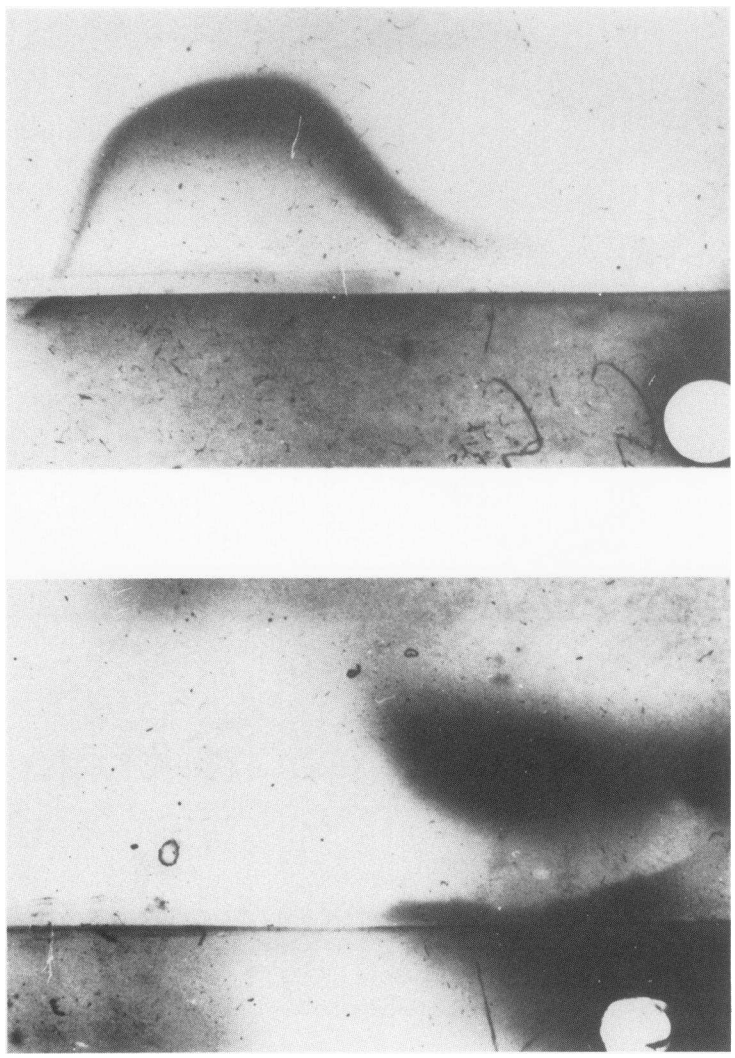

FIGURE 4 Crossed immunoelectrophoresis of concentrated normal and Factor V-deficient plasma. Pooled normal plasma was concentrated (fivefold) by precipitation with 14% PEG. It was then subjected to crossed immunoelectrophoresis (Methods) against polyclonal rabbit anti-human Factor V antiserum (*top*). Crossed immunoelectrophoresis of concentrated plasma deficient in Factor V coagulant activity against polyclonal rabbit anti-human Factor V antiserum (*bottom*).

of this preparation to our immunoadsorbent column gives similar results to the procedure we describe where the steps are reversed but did not improve the yield. Thrombin (0.1 U/ml) hydrolysis of our preparation gave an 18-fold activation somewhat less than that of Kane and Majerus (4) who found a 25–30-fold increase with a much higher thrombin concentration (2 U/ml).

Katzmann et al. (5) isolated human Factor V by the use of a mouse hybridoma antibody with an overall yield of 28%. Their first two steps were similar to ours using barium citrate and PEG precipitation. We found that we could eliminate the PEG precipitation without change in yield or results although it was convenient for concentrating the Factor V. However, eliminating the barium citrate step led to the appearance of proteolytic degradation products of Factor V, presumably due to thrombin or activated protein C. A 5,100-fold purification obtained in our preparation is similar to that of 3,400 (5) and of 5,800 (4) obtained by procedures using a hybridoma or conventional method, respectively. The conditions of elution from the human

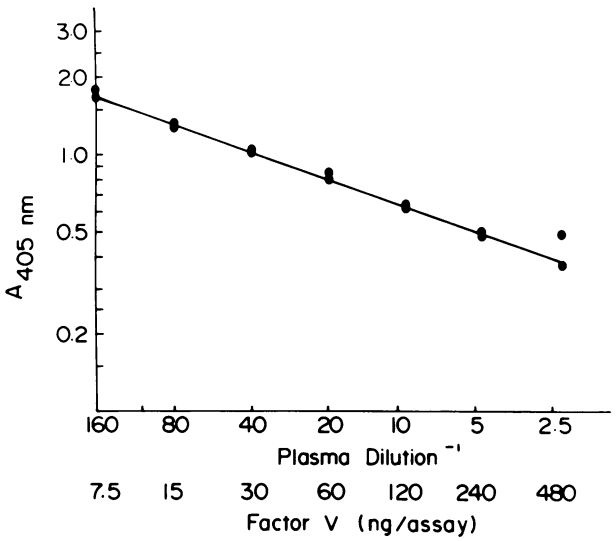

FIGURE 5 Standard curve for competitive ELISA. In this assay representative of 12 such experiments, purified Factor V (100 ng/200 μl) was adsorbed to the surface. The plates were then reacted with rabbit antiserum to Factor V in the presence of different amounts of Factor V (nanograms per assay) prepared by dilution of pooled normal plasma and expressed as the reciprocal of the dilution (plasma dilution^{-1}) on the abscissa. After washing the amount of rabbit antiserum to Factor V that remains complexed with the solid phase, Factor V was further reacted with alkaline phosphatase-conjugated heterologous antibody against rabbit IgG. The complexed antibody was quantified by measuring the absorbance at 405 nm produced by enzymatic action on a chromogenic substrate in the conjugated antiserum. The absorbance was corrected for nonspecific hydrolysis of the substrate (Methods). Each symbol is the mean of triplicate observations. The straight line was obtained by linear regression of logarithm of the absorbance vs. the logarithm of the Factor V concentration and can be represented as $Y = -0.341 \times -0.493$ with a correlation coefficient, $r = 0.99$.

IgG_4 (λ) antibody differ somewhat from that of the murine monoclonal antibody. Whereas for the latter procedure 1.2 M NaCl was sufficient, we required, in addition, the inclusion of 1.0 M α-methylmannoside. We were also able to obtain similar results by substituting 50% glycerol for the α-methylmannoside, although the flow rate was slower. The requirement for high concentration of a sugar suggests that disruption of other noncovalent interactions beside ionic forces may be important for dissociation from the human antibody. Nevertheless, the elution conditions are much milder than those for a polyclonal heterologous antibody, which usually require low pH or a denaturing solvent. The mild conditions are more typical of monoclonal antibodies, which generally exhibit lower affinity for antigens than do polyclonal antibodies.

Although the human antibody is of restricted heterogeneity (IgG_4, λ) no rigorous evidence has been provided that it is strictly monoclonal. The presence of three components on isoelectric focusing (6) suggests, but does not prove, that more than one antibody may be present, since Williamson (14) has indicated that monoclonal antibodies may show microheterogeneity on isoelectric focusing. An interaction involving the carbohydrate of Factor V and the immunoglobulin may be important to the binding to the immunoglobulin. The use of both high salt and sugar in the elution may be applicable to the purification of Factor VIII coagulant protein using naturally occurring human antibodies. Some of the human antibodies against this protein component contain a single type of heavy and light chain (15). The high yield and simplicity of this purification procedure allows purification of Factor V for further biochemical and immunochemical studies.

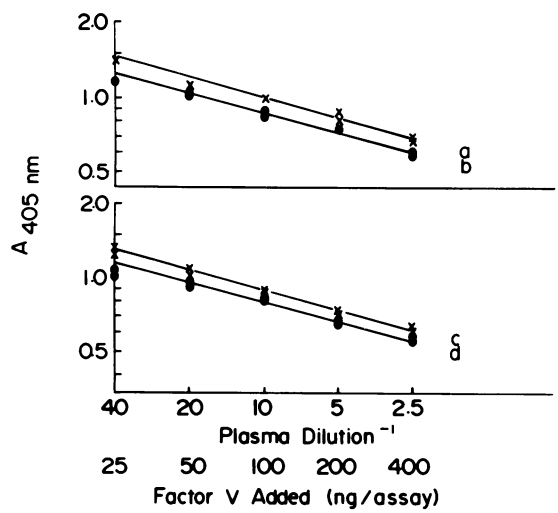

FIGURE 6 Dose-response curves of competitive ELISA. Plasma rendered deficient in Factor V by an immunoabsorbent (*a*, *b*) or plasma from a patient with congenital Factor V deficiency with <0.01 μg/ml antigen (*c*, *d*) were each reconstituted with different amounts of pooled normal plasma (*a*, *c*) or purified Factor V (*b*, *d*). The reconstituted plasma was assayed for Factor V antigen in an ELISA performed as described in Methods. The straight lines obtained as in Fig. 5 can be represented as (*a*) $Y = -0.258 \times -0.264$ and (*b*) $Y = 0.267 \times -0.324$ for the Factor V-immunodeficient plasma reconstituted with normal plasma and purified Factor V, respectively, and (*c*) $Y = -0.257 \times 0.315$ and (*d*) $Y = -0.260 \times -0.361$ for the congenital Factor V-deficient plasma reconstituted with pooled normal plasma (expressed as reciprocal of the dilution) and purified Factor V (expressed as nanograms per assay), respectively. Each point is the mean of triplicate determinations. These assays were performed with a different conjugated antiserum than in Fig. 5 accounting for the different slopes in Fig. 5 and Fig. 6.

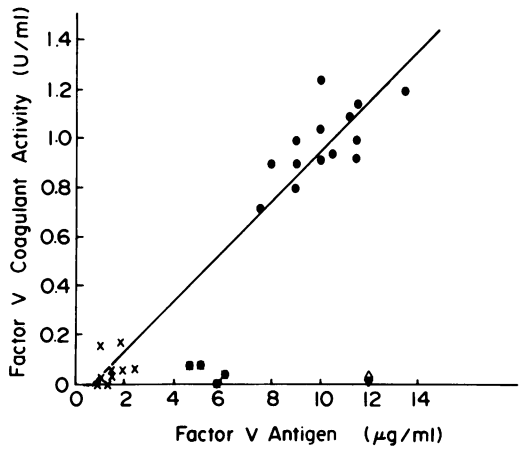

FIGURE 7 Correlation of Factor V activity and antigen in the plasma of normal individuals and patients with congenital Factor V deficiency. Factor V activity was measured by one-stage clotting assay and Factor V antigen levels were determined by ELISA. The straight line was obtained by linear regression and can be expressed as $Y = 0.104 \times -8.2$. The correlation coefficient ($r = 0.97$, $P < 0.01$) was obtained with the values obtained with 14 normal individuals (●) and 10 Factor V congenital deficiencies (×). Four patient plasmas showed low biological activity but moderate concentrations of Factor V antigen (⊠). Heat-inactivated plasma (△) and EDTA-treated plasma (▽) are also represented in this figure. Each patient was assayed at three dilutions, each in triplicate on at least two different occasions.

We have produced a monospecific antiserum to the purified protein that precipitates a single component in plasma with identical electrophoretic migration to purified Factor V. This antiserum has allowed development of an ELISA for human Factor V. The antibody was able to react with the surface-bound Factor V and a second enzyme-labeled antibody was used to quantify the amount of bound rabbit antibody. Factor V, whether in plasma collected in ordinary anticoagulants or in purified samples, was quantified identically in this assay. The normal range of Factor V antigen in human plasma was 7.5–13.5 μg/ml, which is similar to the range of 4 to 14 μg/ml obtained by radioimmunoassay (16). The antiserum can detect Factor V antigen despite inactivation of the coagulant activity by heat, Chelation of calcium known to be essential for Factor V activity (17) also does not alter the antigen expression.

The polyclonal rabbit antiserum contains antibodies against at least two different epitopes. The antigenic determinant(s) recognized by the rabbit antiserum in the ELISA procedure includes domains of the Factor V molecules that are not required for the biological activity of the molecule, since Factor V inactivated nonproteolytically (EDTA or heat) and genetically abnormal Factor V molecules can be identified. In contrast, the epitope(s) recognized by the neutralizing antibody in the rabbit antiserum appear to include the biologically active portions of the molecule. In contrast, the "monoclonal" human antibody does not precipitate human Factor V but can neutralize its activity, suggesting that it recognizes domain(s) of the molecules required for biological activity.

Some neutralizing antibodies to coagulation proteins such as Factor X (18) can detect abnormal molecules with low coagulant activity and either normal or modestly reduced antigen. However, the inability of previous investigators using human antibodies to Factor V to detect human mutants with abnormal Factor V protein (19, 20) may have been due to the similarity of these antibodies to the human antibody used in this study.

This study has identified a subset of patients with Factor V deficiency with relatively high Factor V antigen, suggesting that these individuals possess a mutant nonfunctional Factor V molecule. Previously there are no well documented proven reports of human abnormal Factor V molecules. A single study (21), which claimed to be unable to detect abnormal Factor V in several patients, used a heterologous antibody raised to what is now known to be a preparation of Factor V, which was probably not homogeneous. Tracy et al. (16) using a radioimmunoassay did not detect Factor V antigen in two patients studied with Factor V deficiency. The presence of Factor V antigen and activity in similar concentrations in the one patient with combined Factor V and VIII deficiencies is consistent with current concept of the disease as a deficiency of an inhibitor of activated protein C (22).

It is interesting, to note that the Factor V antigen in the variant form of Factor V deficiency, is reduced in concentration compared with the normal molecule ranging from 40 to 55% of normal. This observation is in accord with studies of other abnormal proteins, such as fibrinogen (23), where the concentration of the abnormal protein is frequently less than that of the normal molecules usually due to decreased synthesis. Alternatively, the Factor V might be catabolyzed more rapidly in the circulation, as has been reported for two hypodysfibrinogenemias (24, 25). The structural and functional abnormalities in these individuals with variant Factor V is currently under scrutiny in our laboratory.

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REFERENCES

1. Colman, R. W. 1976. Factor V. *Prog. Hemostasis Thromb.* 3:109–143.
2. Nesheim, M. E., J. A. Katzmann, P. A. Tracy, and K. Mann. 1981. Factor V. *Methods Enzymol.* 80:249–274.
3. Dahlbäck, B. Human coagulation Factor V. 1980. Purification and thrombin-catalyzed activation. *J. Clin. Invest.* 66:583–591.
4. Kane, W. H., and P. W. Majerus. 1981. Purification and characterization of human coagulation Factor V. *J. Biol. Chem.* 256:1002–1007.
5. Katzman, J. A., H. E. Nesheim, L. S. Hibbard, and K. G. Mann. 1981. Isolation of functional human coagulation Factor V by using a hybridoma antibody. *Proc. Natl. Acad. Sci. USA.* 78:162–166.
6. Hurtubise, P. E., M. C. Coots, D. J. Jacob, A. F. Muhlewa, and H. I. Glueck. 1979. A monoclonal IgG_4 (λ) with Factor V inhibitory activity. *J. Immunol.* 122:2119–2221.
7. March, S. C., I. Parikh, and P. Cautrecasas. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* 60:149–152.
8. Ware, A. G., R. C. Murphy, and W. H. Seegers. 1947. The function of Ac-globulin in blood clotting. *Science (Wash. DC).* 106:618–619.
9. Engvall, E. 1980. Enzyme immunoassay ELISA and EMIT. *Methods Enzymol.* 70:419–439.
10. Ouchterlony, O., and L. A. Nilsson. 1978. Immunodiffusion and immunoelectrophoresis. *In* Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications, Philadelphia.
11. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4412.
12. Chesney, C. M., D. Pifer, and R. W. Colman. 1981. Subcellular localization and secretion of Factor V from human platelets. *Proc. Natl. Acad. Sci. USA.* 78:5180–5184.
13. Suzuki, K., B. Dahlbäck, and J. Stenflo. 1982. Thrombin-catalyzed activation of human coagulation Factor V. *J. Biol Chem.* 257:6556–6564.
14. Williamson, A. R. 1978. Isoelectric focusing of immunoglobulins. *In* Handbook of Experimental Immunology. D. M. Wier, editor. Blackwell Scientific, Philadelphia. 1:
15. Robboy, S. J., E. J. Lewis, P. H. Schur, and R. W. Colman. 1970. Circulating anticoagulants to factor VIII. *Am. J. Med.* 59:742–752.
16. Tracy, P. B., X. Eidell, E. J. W. Bowie, and K. G. Mann. 1982. Radioimmunoassay of factor V in human plasma and platelets. *Blood.* 60:59–63.
17. Greenquest, A. C., and R. W. Colman. 1975. Bovine factor V: a calcium-containing metalloprotein. *Blood.* 46:769–782.
18. Denson, K. W. E., A. Lurie, F. DeCataldo, and P. M. Mannuci. 1965. The factor X defect. Recognition of abnormal forms of factor X. *Br. J. Haematol.* 18:317–327.
19. Feinstein, D. E., S. I. Rapaport, W. G. McGehee, and M. J. Patch. 1970. Factor V anticoagulants: clinical, biochemical, and immunological observations. *J. Clin. Invest.* 49:1578–1588.
20. Fratantoni, J. C., M. Hilgartner, and R. L. Nachman. 1972. Nature of the defect in congenital factor V deficiency: study in a patient with an acquired circulating anticoagulant. *Blood.* 39:751–759.
21. Giddings, J. C., S. A. M. Shearn, and A. L. Bloom. 1975. The immunological localization of factor V in human tissue. *Br. J. Haematol.* 29:57–65.
22. Marlar, R. V., and J. H. Griffin. 1980. Deficiency of protein C inhibitor in combined Factor V/VIII deficiency disease. *J. Clin. Invest.* 66:1186–1189.
23. Beck, E. A. 1982. Congenital disorder of fibrin formation and stablization. *In* Hemostasis and Thrombosis. R. W. Colman, V. H. Marder, J. Hirsch, and E. Salzman, editors. J. B. Lipincott, Co., Philadelphia. 185–209.
24. Martinez, J., R. R. Holburn, S. Shapiro, and A. J. Erslev. 1974. Fibrinogen Philadelphia. A hereditary hypodysfibrinogenemia characterized by fibrinogen hypercatabolism. *J. Clin. Invest.* 53:600–611.
25. Gralnick, H. R., B. S. Coller, J. C. Fratantoni, and J. Martinez. 1979. Fibrinogen Bethesda III: a hypodysfibrinogenemia. *Blood.* 53:28–46.