Patients with Congenital Factor V Deficiency have Decreased Factor X_a Binding Sites on their Platelets

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ABSTRACT Human platelets have binding sites for plasma coagulation Factor X_a that are available only after the platelet release reaction. Platelets from 15 normal donors bound 216±52 (SD) molecules of Factor X_a per platelet. The association of Factor X_a with its platelet surface receptor results in a 300,000-fold increase in the catalytic activity of Factor X_a in forming thrombin from prothrombin. The turnover number for platelet-bound Factor X_a was 1,850±460 mol thrombin/ml per min per mol Factor X_a in experiments with platelets from 15 normal donors. Platelets from five patients with varying degrees of Factor V deficiency were investigated to determine whether or not coagulation Factor V participates in either aspect of the Factor X_a-platelet interaction. The binding of Factor X_a to platelets and the accompanying increase in rate of thrombin formation were either reduced in parallel or absent in each case with values ranging from 0 to 45% of control values. The apparent affinity of Factor X_a from Factor V-deficient patients was normal when platelet binding was detected. The supernate from thrombin-treated control platelets, which contains Factor V activity, corrected the Factor X_a binding deficiency of the platelets from three patients tested. Immunoreactive Factor V determined with an homologous antibody corresponded to the functional Factor V activity of platelets from one patient with Factor V deficiency, suggesting that the patient's platelets have a decreased amount of normal Factor V.

The ability of platelets from the patients to bind Factor X_a and increase the rate of thrombin formation correlated with the severity of each patient's bleeding disorder better than the plasma level of Factor V. The results indicate that Factor V is required for the Factor X_a-platelet interaction and that thrombin formation at the platelet surface is important in normal hemostasis.

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INTRODUCTION

We have previously reported the appearance of binding sites specific for Factor X_a on the surface of human platelets after the platelet release reaction (1). The Factor X_a-platelet binding reaction has the following characteristics (1–2): (*a*) there are 200 sites per platelet with an apparent association constant of $3 \times 10^{10} M^{-1}$; (*b*) binding is rapidly reversible suggesting that it occurs at the platelet surface; (*c*) calcium is required for the reaction; (*d*) closely related coagulation factors, such as prothrombin, thrombin, Factor X, diisopropylphosphoryl Factor X_a, Factor IX, and Factor IX_a do not displace Factor X_a from the binding sites; (*e*) bound Factor X_a is 300,000-fold more active than free Factor X_a in converting prothrombin to thrombin; (*f*) Factor X_a binding sites are not present in intact human lymphocytes or erythrocytes.

Factor V is known to cause an increase in the enzymatic conversion of prothrombin to thrombin by Factor X_a, and Factor V binds to agarose-linked Factor X_a in the presence of calcium ions (3). Factor V activity associated with isolated platelets was reported thirty years ago (4–5). The activity is contained within platelets until stimulation (e.g., collagen) or disruption (freeze thawing) causes its release (6, 7). Therefore, it seemed likely that Factor V might be involved in the interaction of Factor X_a with platelets. We have demonstrated (2) that an antibody specific for Factor V (2, 8) blocks both Factor X_a binding to platelets and the accompanying increase in the rate of thrombin formation. We now report that Factor V from platelets, which is available only after the release reaction, is essential for Factor X_a binding based on experiments with platelets from five patients with varying degrees of congenital Factor V deficiency.

METHODS

Sources for materials and methods for preparing and assaying prothrombin, thrombin, Factor X_a, and control and anti-Factor

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V human immunoglobulin (Ig)G, and for iodinating Factor X_a are described elsewhere (1, 2). The methods for measuring thrombin generation and ^{125}I-Factor X_a binding to washed platelets are also detailed in these reports (1, 2). Thrombin units were determined by National Institutes of Health standard B-3. The Factor X_a used in these experiments formed 300 U thrombin/min per μg Factor X_a when assayed with bovine Factor V_a and phospholipids.

Platelets from Factor V-deficient patients. Because all five patients were seen in distant cities, the time between blood drawing and the actual experiments varied. In every instance blood was drawn from the control (J. M.) at the same time and was handled in exactly the same manner. There was no significant difference in the values for the control between experiments, and these values were compared with those obtained with platelets from 14 other normal donors. All equipment and materials necessary for the experiments were taken to Muncie, Ind. to study the first patient (E. N.), and one set of experiments was carried out as soon as the platelets were obtained. Freshly isolated platelets were also flown back to our laboratory (5-h delay). On a subsequent occasion E. N. was studied in St. Louis and tested there. Because the results were identical with fresh platelets in Muncie or St. Louis and with platelets stored during transit, platelets from the remaining four patients were tested in St. Louis. We determined that isolated platelets are stable with respect to their Factor X_a binding properties and rates of thrombin formation for at least 16 h when stored at a concentration of 10^9/ml in the phosphate buffer used for washing them free of plasma (0.113 M sodium chloride, 4.3 mM dipotassium phosphate, 4.3 mM disodium phosphate, 24.4 mM monosodium phosphate, and 1 mg/ml glucose). Platelets from the second patient (K. S.) and the control were isolated, tested immediately for their ability to increase the rate of thrombin formation by Factor X_a, and flown to St. Louis (11-h delay) for further experiments where similar rates of thrombin formation were observed. Later we determined that blood samples collected in 5.7 mM EDTA in plastic syringes can be left at room temperature for at least 8 h with no apparent effect on the final yield or characteristics of the isolated platelets. Therefore whole blood samples from the final three patients (S. G., D. S., and R. G.) and the control were flown to St. Louis (4-h delay).

Plasma levels of all coagulation factors other than Factor V were normal for E. N., K. S., and R. G. S. G. and D. S. have combined Factor V and Factor VIII deficiency with plasma Factor VIII levels of 15 and 17%, respectively, as measured in the laboratory of Dr. H. Joist, Washington University Thrombosis Center Core Hemostasis Laboratory.

Factor V assay. We have reported (2) a two-stage assay for Factor V (V_a) that measures the rate of thrombin formation with homogeneous human prothrombin and Factor X_a. A more convenient one-stage procedure has also been developed that differs only by addition of highly purified fibrinogen. The assay mixture contains 50 μl of 10 mg fibrinogen/ml, 50 μl of 100 μg human prothrombin and 250 μg rabbit brain cephalin/ml, 50 μl of 25 ng human Factor X_a/ml, and 50 μl of sample. All reagents are in 0.15 M NaCl, 0.02 M Tris HCl, containing 5 mM trisodium citrate, pH 7.4. The sample and the Factor X_a solutions also have 10 mg recrystallized and lyophilized bovine serum albumin/ml. The reaction is initiated by addition of 50 μl of 40 mM $CaCl_2$.

Fibrinogen preparation. Commercially available fibrinogen preparations are not suitable for the one-stage Factor V assay because they result in short blank times (presumably because of contamination with small amounts of Factor V) that limits the sensitivity. Human fibrinogen was prepared by the method of Straughn and Wagner (9). The purification scheme was modified by redissolving the fibrinogen in 10% of the original plasma volume after the first 2.0 M β-alanine precipitation, treating with 5 mM diisopropylfluorophosphate for 30 min, and adding buffer containing 1 mM benzamidine hydrochloride (Sigma Chemical Co., St. Louis, Mo.), 0.1 mM phenylmethylsulfonylfluoride (Sigma Chemical Co.), and 10 mM 6-aminohexanoic acid (Eastman Chemical Products, Inc., Kingsport, Tenn.) to the original plasma volume. The final product was stored at −50°C in 0.15 M sodium chloride containing 5 mM trisodium citrate and 20 mM Tris-HCl, pH 7.5. The blank time for the Factor V assay with this fibrinogen was >5 min.

RESULTS

Factor V assay. Fig. 1 shows a standard curve for the Factor V assay with dilutions of purified thrombin-activated bovine Factor V (2). The usefulness of the assay is indicated by the 100-fold range (0.3–30 mU/ml) of Factor V activity measured. Possible errors in measurements of Factor V activity that might result from activation of serine proteases other than thrombin are minimized in this defined reaction mixture. The assay is very reproducible with variations of only ±5 s at the longest clotting time. Dilutions of pooled normal human plasma (76 donors) result in a concentration curve of identical slope. We estimate that human plasma has 3.0–3.5 μg of Factor V/ml relative to the bovine preparation we used as a standard, because plasma samples diluted 1,000-fold in the final assay method result in a clotting time of ≅200 s, the mid-range of the standard curve. We do not know the relative activity of bovine vs. human Factor V nor are we certain that the bovine Factor V is native and homo-

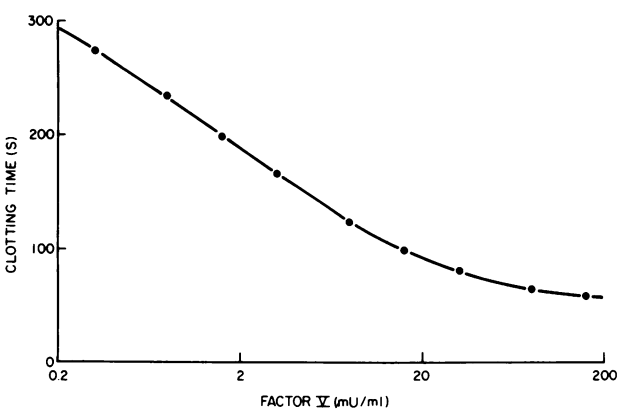

FIGURE 1 Factor V assay. The activity of purified bovine Factor V_a (compared to normal human plasma defined as 1 U/ml) in the reaction mixture is indicated on the abscissa. The thrombin-activated Factor V used in this experiment had an activity of 350 U/A_{280}. Final concentrations of the other reactants were: human prothrombin, 20 μg/ml; human Factor X_a, 5 ng/ml; rabbit brain cephalin, 100 μg/ml; bovine fibrinogen, 2 mg/ml; calcium chloride, 8 mM. Samples and reagent proteins were in 0.15 M sodium chloride, 5 mM trisodium citrate, and 20 mM Tris-HCl, pH 7.5. The reactions were initiated with 40 mM calcium chloride. Blank time was >300 s.

geneous, so this estimate may be erroneous. The bovine Factor V standard is stable (2) and was used to estimate the quantity of platelet Factor V as outlined below. We do not imply that the platelet Factor V corresponds to bovine Factor V in absolute quantity.

Thrombin-induced [^{14}C]serotonin release from platelets. Because both Factor X_a binding and enhanced rate of thrombin formation occur only after platelets undergo the release reaction, platelets from each patient were tested for their ability to secrete [^{14}C]serotonin in response to low levels of thrombin. Control platelets (J. M.) consistently underwent maximal release in 2 min with 0.5 U thrombin/ml with 50% of maximal release at 0.045 U thrombin/ml. Platelets from all five patients also showed maximal release with 0.5 U thrombin/ml with 50% of maximal release at values of 0.022 (E. N.), 0.024 (K. S.), 0.025 (S. G.), 0.040 (D. S.), and 0.020 (R. G.) U thrombin/ml. These values are all within the range observed for platelets from normal donors (0.01–0.05 U thrombin/ml).

Rates of thrombin formation by Factor X_a in the presence of platelets. Fig. 2 shows the increase in thrombin concentration with time after the addition of Factor X_a to reaction mixtures containing platelets, prothrombin, Ca^{++}, and 0.5 U thrombin/ml. The thrombin in the initial mixture was used to cause the platelet release reaction. Data for the control and for two patients are shown in Fig. 2. The values shown for the control were determined during experiments with platelets from K. S. but are very similar to values obtained while testing platelets from E. N. The rate of thrombin formation without added platelets was 0.014 U thrombin/ml per min when 1 μg Factor X_a was added. Control platelets increased this rate to 3.00 U thrombin/ml per min when only 5 ng Factor X_a/ml was tested

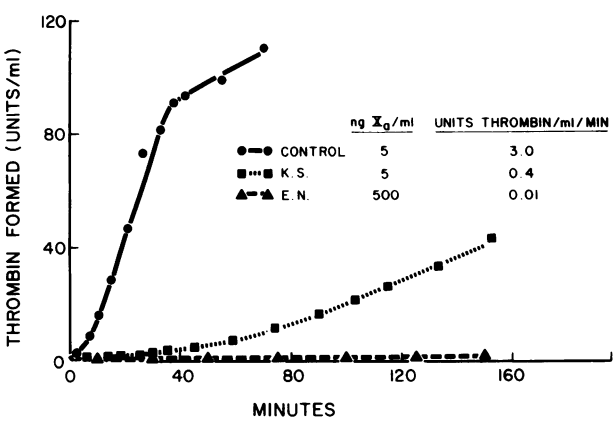

FIGURE 2 Platelet-accelerated thrombin formation. Reaction mixtures initially contained 70 μg human prothrombin/ml, 10^8 platelets/ml, 0.5 U human thrombin/ml, and human Factor X_a as indicated in 0.15 M sodium chloride, 2.5 mM calcium chloride, 5 mg bovine serum albumin/ml, and 20 mM Tris-HCl, pH 7.3.

(5 ng Factor X_a is enough to produce 80–90% saturation of platelet Factor X_a binding sites on 10^8 platelets). Platelets from E. N., however, caused very little if any acceleration of the rate because 5 ng Factor X_a/ml resulted in undetectable thrombin formation and 0.5 μg Factor X_a ml produced only 0.01 U thrombin/ml per min.

Platelets from the patient K. S. increased thrombin formation 13% as well as control platelets, the final rate being 0.39 U thrombin/ml per min when 5 ng Factor X_a/ml was added. The time required to reach the linear rate of thrombin formation was also increased with platelets from K. S. These reactions were all carried out in buffer containing 20 mM Tris at pH 7.3. Platelets produce acid metabolites and Tris has little buffering capacity for hydrogen ions at this pH. After completion of these initial experiments we realized that the actual pH in the reaction mixtures was more acidic than 7.3. We have reported that the rate of thrombin formation by Factor X_a decreases as the pH is changed from 8.5 to 6.1, with effects being most pronounced below 7 (2). The pH of the buffer was adjusted for studies with platelets from the remaining patients so that the actual pH in the reaction mixtures was 7.4. The rates measured after addition of 5 ng Factor X_a/ml were: control, 5.6; S. G., 2.5 (44%); D. S., 2.0 (36%); and R. G., 0.05 (1%) U thrombin/ml per min. The percentage values in parentheses relate the patient values to the control. Except for the experiment with R. G.'s platelets, these rates were determined after they became constant, which took proportionally longer in the cases where the final rate was lower. The rate for R. G.'s platelets, undetectable for 30 min, was measured at 3 h and may have been still increasing slightly.

14 normal donors (seven male and seven female) were divided into two groups that were studied on separate days. The control (J. M.) was the eighth member of each group. Isolated platelets were tested for their ability to increase thrombin formation by 5 ng/ml of Factor X_a. The mean was found to be 5.63±0.92 (SD) U thrombin/ml per min); the value for the control was 5.25 in both of these experiments.

Binding of ^{125}I-Factor X_a to released platelets. Steady-state binding of ^{125}I-labeled Factor X_a to platelets was investigated at concentrations from 0.5 to 20 ng/ml. As previously described (2), the time required to reach maximal binding increases as the concentrations of ^{125}I-Factor X_a decreases. Longer times were also necessary for optimal binding at a given ^{125}I-Factor X_a concentration to platelets from patients with decreased Factor V than for binding to control platelets. The reason why the time necessary to reach apparent steady-state binding (and maximal thrombin formation) depends upon both Factors X_a and V concentrations remains obscure. Binding of ^{125}I-Factor X_a decreases

when prothrombin has mostly been converted to thrombin, so a time-course from 15 to 120 min was followed with a nonspecific binding correction, which was <10% of total binding (duplicate reaction mixture plus 100-fold excess unlabeled Factor X_a) (2), for each data point (and for each platelet preparation) at each level of ^{125}I-Factor X_a. The final concentration dependence of ^{125}I-Factor X_a binding reported for each platelet preparation was constructed from the points of maximal specific binding.

Platelets from E. N. demonstrated no specific ^{125}I-Factor X_a binding when concentrations from 1 ng to 1 μg/ml were tested in incubations for up to 3 h.

Fig. 3 shows ^{125}I-Factor X_a binding to platelets from the control and from K. S. Analysis of the data by a double reciprocal plot showed that the number of receptors rather than the apparent association constant ($3.1 \times 10^{10} M^{-1}$) is reduced with platelets from K. S., suggesting a deficiency of normal Factor V molecules. In this experiment, control platelets bound 1.17 ng ^{125}I-Factor X_a/10^8 platelets whereas K. S. platelets bound 15% of that value (0.18 ng ^{125}I-Factor X_a/10^8 platelets).

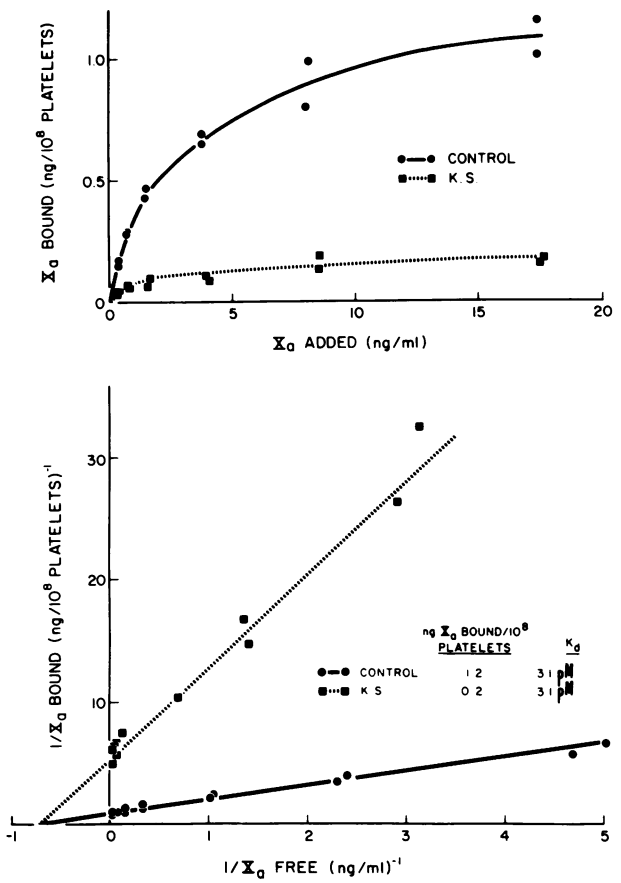

FIGURE 3 Steady-state binding of ^{125}I-Factor X_a to control and K. S. platelets. Top: Concentration dependence of ^{125}I-Factor X_a binding. Reaction mixtures were as in Fig. 2 except that variable amounts of ^{125}I-Factor X_a were added instead of unlabeled Factor X_a. Maximum specific binding for each ^{125}I-Factor X_a were added instead of unlabeled Factor X_a. Maximum specific binding for each ^{125}I-Factor X_a concentration was determined as discussed in the text. Bottom: Double-reciprocal plot of the binding data.

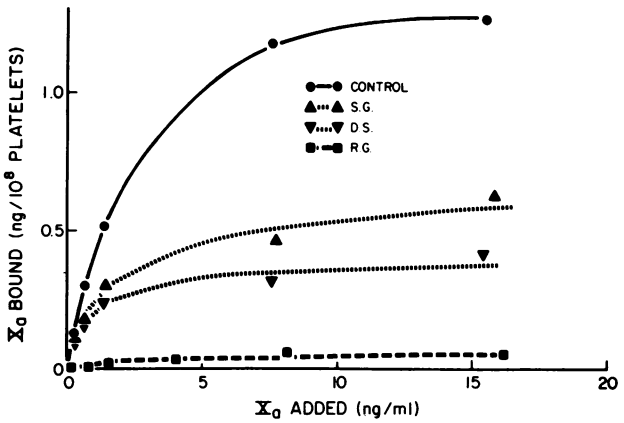

FIGURE 4 Steady-state binding of ^{125}I-Factor X_a to control, S. G., D. S., and R. G. platelets. Reaction mixtures were as in Fig. 3. Each point is the average of duplicate determinations.

Binding data for the remaining three patients is shown in Fig. 4. When these data were analyzed by double-reciprocal plots (not shown) it was found that binding was reduced relative to the control (1.22 ng/10^8 platelets) to 45% for S. G. (0.55 ng/10^8 platelets) and 34% for D. S. (0.41 ng/10^8 platelets). As found with platelets from K. S., binding to platelets from S. G. and D. S. showed the same apparent association constant ($3.3 \times 10^{10} M^{-1}$). It was not possible to measure binding accurately with platelets from R. G. because specific binding was a small fraction (<10%) of total binding and because we could not demonstrate saturation of the small amount of specific binding with respect to time or concentration of ^{125}I-X_a. The maximal specific binding observed was <4% (0.05 ng/10^8 platelets) of the control for ^{125}I-Factor X_a concentrations up to 20 ng/ml incubated for 120 min.

Binding data for platelets from the 14 normal donors averaged 1.65±0.04 ng ^{125}I-Factor X_a/10^8 platelets at saturation; in these experiments platelets from the control bound 1.15 ng. The enhancement of Factor X_a catalytic activity by binding to platelets was calculated for each control by dividing the rate of thrombin formation by the observed Factor X_a binding at saturation. The mean turnover number was 1,850±460 mol thrombin formed/min per mol Factor X_a. This reflects a mean enhancement of Factor X_a activity of 300,000-fold compared to Factor X_a in solution (turnover number 0.006 mol thrombin formed/min per mol Factor X_a) (2). These

turnover numbers may not be strictly comparable because the experiments are done under different conditions. Thus Factor X_a activity in solution is measured with 10–100 ng Factor X_a/ml, whereas bound to the platelet only 1–2 ng Factor X_a/ml are present (2).

Correction of the Factor X_a binding defect of platelets from Factor V-deficient platelets by the supernate from thrombin-treated control platelets. In preliminary experiments we observed that treatment of platelets at high concentrations (2×10^9/ml) with thrombin 5 U/ml followed by immediate sedimentation of the platelets through oil in a microfuge at 12,000 *g* for 2 min yielded a supernatant fraction containing factor V activity (0.5 U/10^8 platelets, where normal human plasma has 1 U/ml). Østerud et al. have shown that platelet Factor V is activated (probably Factor V_a) and thus the platelet Factor V activity reflects less mass of Factor V than does the assay of Factor V in plasma. Indeed the assay of dilutions of platelet Factor V (?V_a) in our assay gave a slope similar to the bovine Factor V_a studied.

Platelets from S. G., D. S., R. G., and the control were incubated at 10^8/ml in reaction mixtures containing prothrombin, 5 ng ^{125}I-Factor X_a/ml and the fraction that contains platelet Factor V obtained from normal platelets as described above (1.0 U Factor V) from 2×10^8 control platelets. Nonspecific binding was determined by addition of 1 μg unlabeled Factor X_a/ml. Specific binding, measured in duplicate after 15 min, expressed as ng ^{125}I-Factor X_a bound/10^8 platelets were: S. G., 1.21; D. S., 0.98; R. G., 1.04; and control, 1.28. Thus, in this experiment Factor X_a binding to platelets from three Factor V-deficient patients was corrected by the addition of Factor V containing supernate from control platelets, even though binding to control platelets was not increased by the same treatment.

We have determined that Factor X_a binding to normal platelets is increased <25% when higher concentrations of ^{125}I-Factor X_a (25 ng/ml) and the supernatant platelet Factor V from 10^9 released platelets (5 U Factor V) are added to 10^8 platelets. The results suggest that Factor V released from platelets can participate in Factor X_a binding at the platelet surface and that the number of binding sites is limited by the component that binds Factor V-X_a complex rather than Factor V itself. The experiment also indicates that S. G., D. S., and R. G. do not lack the platelet component that interacts with platelet Factor V-X_a complex but rather are missing the Factor V itself.

Binding of bovine Factor V_a to platelets from a Factor V-deficient patient. The experiments presented above suggest, but do not prove, that Factor V_a itself provides the binding site on platelets for Factor X_a. This could be established directly by studies measuring binding of ^{125}I-Factor V_a to platelets. Unfortunately, Factor V_a has not yet been isolated from Factor V nor is it established what part of the Factor V molecule is contained in Factor V_a. We used bovine Factor V_a (thrombin-treated bovine Factor V) for the experiment shown in Table I. The material was labeled to sp act 135 cpm/ng although we cannot use this figure to determine the actual amount of Factor V_a bound to platelets because we do not know the specific activity nor size of the active component. However, as shown in Table I ^{125}I-Factor V_a does bind to platelets only after thrombin treatment. The binding requires calcium ions and is displaced by 10 μg/ml unlabeled Factor V_a. The binding was increased threefold in the presence of Factor X_a suggesting that Factors X_a and V_a form a complex at the platelet surface. It was not possible to measure sequentially Factors V_a and X_a binding because thrombin-treated platelets aggregate when

TABLE I

Binding of Bovine Factor V_a to Platelets from a Factor V-Deficient Patient

Additions	^{125}I-Factor V_a	^{125}I-Factor V_a plus unlabeled Factor V_a	Specific binding (1-2)
		cpm bound/10^8 platelets	
Thrombin-treated platelets	4,610	1,310	3,300
Thrombin-treated platelets plus Factor X_a (10 ng/ml)	10,700	1,580	9,120
Thrombin-treated platelets minus Ca^{++}	948	964	0
Untreated platelets	1,010	—	—

Freshly isolated platelets from E. N. were incubated at 10^8/ml with bovine ^{125}I-Factor V_a (0.2 μg/ml, 135 cpm/ng Factor V), 5 mM $CaCl_2$, 75 μg prothrombin/ml, and where indicated 10 ng Factor X_a/ml and 10 μg unlabeled Factor V_a/ml. Reaction mixtures were incubated 15 min at 25°C and the platelets were collected by sedimentation through oil as described previously (2). Bovine Factor V_a was labeled with the procedure described previously for labeling Factor X_a (2).

sedimented and cannot be resuspended to carry out a two-stage binding assay.

Inhibition of platelet accelerated rate of thrombin formation by an anti-Factor V human IgG. We have previously reported (2) that a highly purified, homogeneous IgG paraprotein isolated from the plasma (provided by Dr. Helen Glueck, University of Cincinnati) of a patient with an acquired inhibitor to Factor V (8), causes reduced Factor X_a binding and thrombin formation when incubated with thrombin-treated platelets. The antibody is specific for Factor V and cross-reacts with purified bovine Factor V. The inhibition of both binding and thrombin formation is overcome with time after addition of Factor X_a, presumably because the affinity of Factor X_a is greater than that of the antibody. IgG from normal human plasma has no effect on the Factor X_a-platelet interaction even at 1,000-fold higher concentrations.

We measured the effect of the anti-Factor V IgG by incubating it at varying concentrations (0.05–2.0 μg/ml) for 20 min with thrombin-treated platelets. After the addition of prothrombin (70 μg/ml) and Factor X_a (5 ng/ml) we measured thrombin formation with time. The rate of thrombin formation was determined at the earliest time a particular platelet preparation reached the maximal rate in the absence of added inhibitor.

The results for control and K. S. platelets are shown in Fig. 5. It appears that the patient's platelets do not have a significant amount of nonfunctional cross-reactive Factor V because only about one-sixth the concentration of anti-Factor V antibody was necessary to achieve the same initial degree of inhibition relative to control platelets. Feinstein et al. were also unable to detect cross-reacting material in plasma from the same patient (K. H. in their study) with a different homologous anti-Factor V antibody (10).

Plasma Factor V. Plasma from each patient and the control was assayed for Factor V. The control plasma (J. M.) had 85% of the activity of pooled normal plasma and was the same for each experiment. Plasmas from E. N., K. S., and R. G. had no Factor V activity when diluted 1,000-fold and prolonged the blank time of the assay when used at higher concentrations. Plasma samples from S. G. and D. S. had 1.6 and 2.4% of the control level when assayed at fivefold dilution. However, the apparent activity in their plasmas increased slightly with further dilution; 1.9 and 2.7% at 12.5-fold, 2.2 and 3.0% at 25-fold, 2.5 and 3.4% at 50-fold, and 3.1 and 3.8% at 125-fold. No activity was detectable at higher dilutions. The values at the highest dilution were taken as the patients' plasma Factor V levels as shown in Table II.

In mixing experiments all of the plasmas reduced the activity of the control plasma by 10–20%. The nature of these slight inhibitions was not determined, but their presence prevented a straightforward use of the anti-Factor V antibody to check for low levels of plasma cross-reacting material.

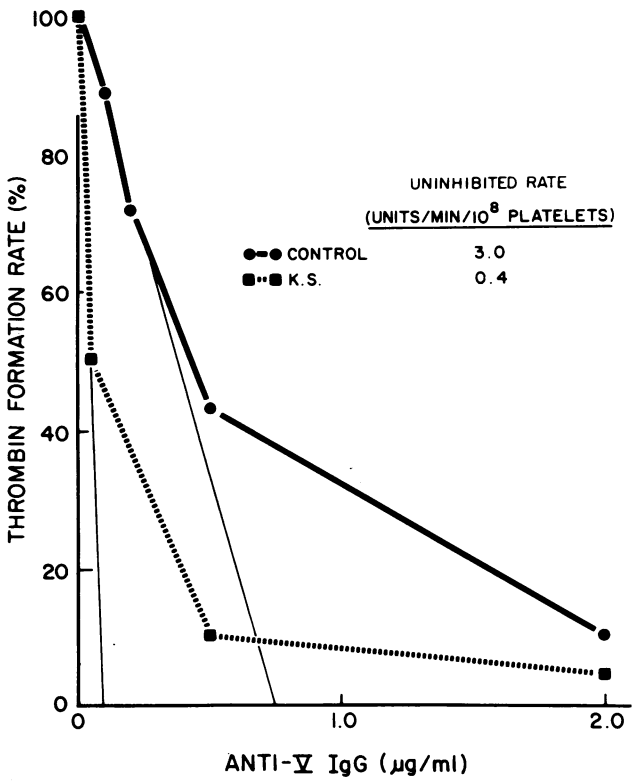

FIGURE 5 Inhibition of thrombin formation by anti-Factor V IgG. Control or K. S. platelets (10^8/ml) were incubated for 20 min with 0.5 U human thrombin/ml, 10 μg normal human IgG/ml, and anti-Factor V IgG as indicated on the abscissa in 0.15 M sodium chloride, 2.5 mM calcium chloride, 5 mg bovine serum albumin/ml, and 20 mM Tris-HCl, pH 7.3. After the addition of 70 μg human prothrombin/ml and 5 ng human Factor X_a/ml, thrombin formation was determined at various times. The rates presented were determined at the time when thrombin formation was maximal in reactions without the Factor V inhibitor. The extrapolated lines approximate the amount of antibody required to inactivate the Factor V in each sample.

Clinical severity of Factor V deficiency. Each patient was interviewed for a history of bleeding problems. Only E. N. and R. G. regarded themselves as "bleeders" and felt that their life styles had been severely restricted by their problem. Both first had trouble at an early age (6–7 yr) and both have required transfusions (1–2 episodes per year) throughout most of their adolescent and adult lives. Both have had frequent bruises, after minor trauma, that healed slowly. In contrast, the remaining three patients, K. S., S. G., and D. S., have had mild problems and do not consider themselves handicapped. Each was diagnosed as having a bleeding problem at age 24 or older. Other than for prophylaxis they have not required transfusions. Episodes of broken bones, a concussion, tooth extractions, childbirth, and surgery with minimal or no

TABLE II
Summary of Factor V Deficiency in Five Patients

Patient	Age	Sex	Bleeding difficulty	Bleeding episodes requiring transfusion	Plasma Factor V	Factor X_a binding to platelets	Platelet-accelerated thrombin formation rate
	yr				*% control*	*% control*	*% control*
E. N.	30	F	Severe	>50	0	0	0
R. G.	39	M	Severe	>50	0	<4	1
K. S.	52	F	Mild	4	0	15	13
D. S.	28	M	Mild	1	4	34	36
S. G.	47	F	Mild	1(?)	3	45	44

bleeding complications were reported. All three noted some increased bruising and one patient (D. S.) volunteered that this was only a problem after aspirin ingestion. A brief summary of our findings with these patients is presented in Table II.

DISCUSSION

Congenital Factor V deficiency is a relatively rare disorder with about 60 cases reported (11) since Owren described his original subject in 1947 (12). The sex distribution is roughly equal. About half of the patients first have severe bleeding episodes early in childhood and the remainder are diagnosed in adult life. Some patients have no bleeding manifestations and were diagnosed during family studies. There is not a good correlation between plasma Factor V levels and clinical severity (11). None of the patients used in the current study have had family studies performed to determine the pattern of inheritance which is presumed to be autosomal recessive.

Mann et al. described Factor V activity associated with platelets in 1947 (4), and Ware et al. reported in 1948 (5) that the platelet factor is "an integral part of the platelets" that behaves like activated plasma Factor V. However, in 1955 Hjort et al. (13) concluded that the Factor V activity of platelets is tightly adsorbed, nonactivated plasma Factor V, and this concept was widely accepted. Recently, Breederveld et al. (6) and Østerud et al. (7) have again suggested that Factor V is actually contained in platelets. Our observations on Factor X_a binding are consistent with this hypothesis because Factor V is essential for binding, which occurs to washed platelets only if the platelet release reaction occurs (1). Furthermore, the correction of the Factor X_a binding defect of platelets from Factor V-deficient platelets by the supernatant fraction from thrombin-treated normal platelets indicates that at least some Factor V is released and can then reassociate at an apparently small number of platelet-surface binding sites. It is also possible, though it seems less likely, that the correction of Factor X_a binding to the platelets of Factor V-deficient patients was the result of something other than the Factor V we find in the supernatant fraction. Østerud et al. (7) report that platelets contain an activator of Factor V, as well as Factor V. If the correction of Factor X_a binding to the platelets from the three patients reported here was a result of a Factor V activator rather than Factor V itself, then the Factor V released from platelets might not participate in Factor X_a binding and the number of sites could be determined by nonactivated Factor V already present on the platelet surface. This question can only be answered with the use of purified Factor V in further experiments with Factor V-deficient platelets. Whether plasma Factor V (V_a) can bind to the platelet surface to generate Factor X_a binding sites could not be determined in our experiments because of a lack of pure human plasma Factor V. We cannot add Factor X_a to platelets in the presence of plasma and measure either binding or thrombin generation because of fibrin formation. It is likely that only Factor V_a can bind to platelets. The Factor V from platelets is probably Factor V_a (7). Studies with bovine factors indicate that Factor V_a, but not Factor V, will bind to Factor X_a (3). Because Factor V_a is at least 60-fold more active than Factor V (14), the mass of Factor V_a in platelets is probably very small compared to Factor V in plasma as suggested previously by immunoassay of platelet and plasma Factor V (7).

All five of the patients in this study have very low plasma Factor V levels relative to the control, but their platelets bind 0–45% of the normal amount of Factor X_a. Lewis and Ferugson (15) also reported a Factor V-deficient patient whose plasma level was zero whereas the platelet level was 5–10% of normal. This might indicate that the activities are distinct molecules or that there is a preferential distribution of Factor V favoring platelets at low total levels. On the other hand, it might simply reflect an underestimate of the patients' plasma activity, as a result of the presence of acquired inhibitors or an abnormal molecule with rapid clearance, for example. Alternatively, we

may underestimate the total amount of Factor V (V_a) in normal platelets. Because only 200 molecules of Factor X_a bind per platelet, only a similar small number of Factor V molecules can participate functionally in assays of Factor X_a binding or thrombin formation. Thus, if normal platelets contain a 10-fold excess of Factor V, then platelets with only 1% of this amount might appear to have 10% by our assays. We did not measure the activity of Factor V released from the Factor V-deficient patients by our coagulation assay except in the case of E. N. who had no detectable activity. However, our results demonstrate that the Factor X_a binding capacity of platelets is a more reliable indicator of the clinical status of patients with Factor V deficiency than the level of plasma Factor V, even when a very sensitive and specific assay is employed for the latter.

The enzymatic activity of Factor X_a is increased 300,000-fold when it is bound at the platelet surface. Thus, it is likely that significant thrombin formation occurs at the platelet surface during normal hemostasis and that platelet surface Factor V activity of platelets is involved. Borchgrevink and Owren (16) have reported that platelet transfusion corrected the bleeding time of a Factor V-deficient patient for a time approaching the life span of platelets ($t_{1/2}$ 5–6 days) without elevating the plasma level of Factor V, whereas plasma transfusion was effective for a much shorter time ($t_{1/2}$ 12–15 h). All five patients presented here have very low plasma Factor V. Yet those whose platelets can bind a significant fraction of the normal amount of Factor X_a have mild bleeding disorders, whereas those whose platelets cannot bind Factor X_a have serious problems. These results not only demonstrate that Factor V is an essential participant in the platelet-Factor X_a interaction, but also underscore the probability that platelet surface thrombin formation is an important event in maintaining normal hemostasis.

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