Characterization of an Acquired Inhibitor to Coagulation Factor V

Antibody Binding to the Second C-Type Domain of Factor V Inhibits the Binding of Factor V to Phosphatidylserine and Neutralizes Procoagulant Activity

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Abstract

Coagulation Factor V is an essential component of the prothrombinase complex, which activates the zymogen prothrombin to thrombin. A patient was described who developed a Factor V inhibitor that neutralized the procoagulant activity of Factor V and resulted in a fatal hemorrhagic diathesis (Coots, M. C., A. F. Muhleman, and H. I. Glueck. 1978. *Am. J. Hematol.* 4:193–206). This inhibitor was shown to be an IgG antibody that bound to the light chain of Factor V. Using a series of light chain deletion mutants, we have found that this antibody binds to the second C-type domain of the light chain. Both inhibitor IgG and Fab fragments rapidly neutralized the procoagulant activity of Factor Va, implying that the neutralization resulted from specific binding to the C2 domain. We have previously demonstrated that deletion of the C2 domain results in loss of procoagulant activity, as well as loss of phosphatidylserine-specific binding. Confirming these results, both inhibitor IgG and Fab fragments interfered with phosphatidylserine-specific binding of Factor V. Conversely, preincubation of Factor Va with procoagulant phospholipids protected the cofactor from inactivation by the inhibitor. Our results suggest that this inhibitor neutralizes the procoagulant activity of Factor Va by interfering with the C2-mediated interaction with phospholipid surfaces, thereby disrupting formation of the prothrombinase complex. (*J. Clin. Invest.* 1992. 90:2340–2347.) Key words: coagulation • factor inhibitor • hemorrhagic diathesis • Factor V • cofactor

Introduction

Acquired coagulation factor inhibitors are pathologic immunoglobulins that specifically bind to coagulation factors and either neutralize their procoagulant activity or result in their clearance from the circulation (1). These inhibitors may develop as alloantibodies in patients who have congenital deficiencies and receive replacement therapy, or, more rarely, as autoantibodies in patients with no known previous factor deficiency. Acquired inhibitors have been most frequently reported in patients with autoimmune disorders or lymphoid malignancies, and during pregnancy (2). Most of these inhibitors are directed against Factor VIII, although patients with inhibitors directed against von Willebrand factor, Factor V, Factor IX, Factor XI, and Factor XIII have been reported (3). Factor V inhibitors are relatively rare and have been most frequently reported in the postsurgical setting and/or in association with aminoglycoside antibiotics (3). Several recent reports, however, have described patients developing Factor V inhibitors after exposure to topical bovine thrombin preparations during cardiovascular or neurosurgical procedures (4, 5). In one report, the topical thrombin preparation was shown to contain bovine Factor V, which may have served as the immunogen in that patient (4).

Although coagulation Factors V and VIII share considerable structural and functional homology (6), the incidence, clinical presentation, and course associated with inhibitors to these two cofactors are quite different (2, 3). The bleeding associated with acquired Factor VIII inhibitors is often severe and life-threatening, with the sudden development of large hematomas, retroperitoneal bleeding, and/or cerebral hemorrhagic events (2). Although some of these inhibitors resolve without specific treatment (7), many require aggressive medical intervention, including plasmapheresis and cytotoxic therapy (1, 8). On the other hand, acquired Factor V inhibitors appear to be more variable in their presentation, with many patients presenting with prolonged clotting times but without clinically significant bleeding. Most Factor V inhibitors are low in titer, and they frequently resolve spontaneously without specific treatment (3). Whether the observed clinical differences between acquired inhibitors to Factors V and VIII are a reflection of the much lower concentration of Factor VIII in plasma (Factor V, 10 μg/ml; Factor VIII, 0.2 μg/ml) (6) or other mechanisms of inhibition is unknown. Since $\sim$ 20% of circulating Factor V is stored in platelet α-granules, it has been suggested that this Factor V may be inaccessible to inhibitors, and thereby account for the more benign course frequently seen in these patients (3). For this reason, platelet transfusions have been recommended as a treatment for hemorrhage in patients with Factor V inhibitors (9), although not all patients respond to this intervention (4, 10). A subset of patients with Factor V inhibitors have had fatal outcomes despite aggressive supportive treatment (10, 11). The best characterized case was reported by Coots et al. (11) in 1978. This inhibitor was shown to be an $IgG_4\lambda$ (12) that bound to the light chain of Factor Va (13, 14), and blocked platelet surface prothrombin activation and Factor Xa binding (13).

Our laboratory is using site-directed mutagenesis to study structure-function relationships in coagulation Factor V. Factor V is an essential component of the prothrombinase com-

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plex, which activates prothrombin to thrombin (6). Analysis of the primary structure of Factor V reveals that it is a mosaic protein containing several types of homologous internal repeats (6) (Fig. 1). We have recently used a series of Factor V deletion mutants to localize a phospholipid binding site in the second C-type domain (15). We have now used these deletion mutants to demonstrate that the inhibitor described by Coots, et al. (11) neutralizes the procoagulant activity of Factor Va by binding to the C2 domain. When we investigated the effect of this inhibitor on the binding of Factor V to phospholipids, we found that the inhibitor blocks phosphatidylserine-specific binding of Factor V. Furthermore, preincubation of Factor Va with rabbit brain cephalin protects the cofactor from inactivation by the inhibitor. These results suggest that this inhibitor neutralizes the procoagulant activity of Factor Va by interfering with the C2-domain mediated binding to anionic phospholipids, thereby disrupting formation of the prothrombinase complex.

Methods

Materials. Restriction enzymes, vectors, and T4 DNA ligase were obtained from Bethesda Research Laboratories (Gaithersberg, MD). Oligonucleotides were synthesized on a Milligen 7500 oligonucleotide synthesizer and purified on oligonucleotide purification cartridges (Applied Biosystems, Inc., Foster City, CA). DNA amplification kits were from Perkin-Elmer Cetus Instruments (Norwalk, CT). DNA sequencing kits (Sequenase Version 2.0) were obtained from U.S. Biochemical Corp. (Cleveland, OH). Purified human plasma Factor V, immunoaffinity-purified polyclonal rabbit anti-human Factor V antibodies, and the Factor V activator from Russell's viper venom (RVV-V)[1] were prepared as described previously (16). Human thrombin, human Factor Xa, and human prothrombin were from Haemotologic Technologies, Inc. (Essex Junction, VT). Murine anti-human IgG subclass-specific monoclonal antibodies and human myeloma proteins were from Zymed Laboratories, Inc. (South San Francisco, CA) and The Binding Site, Inc. (San Diego, CA). L-(α)-phosphatidylserine (bovine brain) was from Avanti Polar Lipids (Birmingham, AL). Rainbow and ^{14}C-labeled molecular weight markers, [^{35}S]methionine, deoxyadenosine 5′-α-[^{35}S]triphosphate, and adenosine 5′-α-[^{32}P]triphosphate were from Amersham Corp. (Arlington Heights, IL). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Factor V inhibitor. Plasma samples from this patient were kindly provided by Dr. P. W. Majerus (Washington University, St. Louis, MO) and Dr. H. I. Glueck (University of Cincinnati Medical Center, Cincinnati, OH). The total IgG fraction was isolated from 0.5 ml of patient plasma by chromatography on Protein A Sepharose. All anti-Factor V activity bound to the protein A, with no inhibitory activity remaining in the nonbinding fraction. The IgG fraction was concentrated using a microconcentrator (Centricon 30; Amicon, Beverly, MA), and final concentration was determined by absorbance at 280 nm (17) and by Bradford analysis using the Bio-Rad Laboratories (Richmond, CA) colorimetric protein assay. Fab fragments were prepared from the total IgG fraction by digestion with immobilized papain (Pierce Chemical Co., Rockford, IL), using the recommended procedure of the manufacturer. The extent of the reaction was monitored by SDS-PAGE (PhastSystem; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Residual intact heavy chain and Fc fragments were removed by chromatography on Protein A Sepharose. The absence of intact heavy chains in the Fab fraction after affinity chromatography was confirmed by SDS-PAGE. The Fab fragments were concentrated and the final concentration determined as for the total IgG fraction. Molarities of the IgG and Fab fractions were determined using molecular masses of 150 kD for IgG and 50 kD for Fab (17). As an IgG control, the total IgG fraction from a 0.5-ml sample of pooled human plasma was isolated by chromatography on Protein A Sepharose, and papain-generated Fab fragments were prepared for the control IgG fraction as described above for the patient IgG.

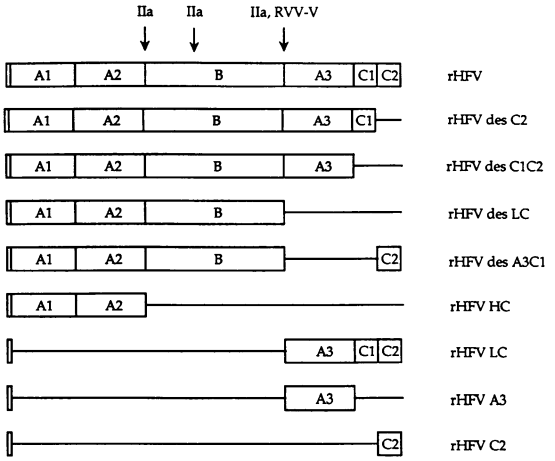

Figure 1. Recombinant human Factor V and light chain deletion mutants. Recombinant Factor V is depicted schematically as a single chain molecule containing three types of internal domains defined by amino acid sequence. The deletion mutants lack domain-size fragments of Factor V and are named accordingly. The modifier 'des' refers to the specific domain(s) deleted. The mutant rHFV des C2 lacks amino acid residues 2037–2196; the mutant rHFV des C1C2 lacks amino acids 1878–2196; the mutant rHFV des LC lacks amino acids 1546–2196; the mutant rHFV des A3C1 lacks amino acids 1546–2036; the mutant rHFV HC lacks amino acids 710–2196; the mutant rHFV LC lacks amino acids 1–1545; the mutant rHFV A3 lacks amino acids 1–1545 and 1878–2196; and the mutant rHFV C2 lacks amino acids 1–2036. The thrombin cleavage sites at arginine residues 709, 1018, and 1545 are indicated by *IIa*, and the single RVV-V cleavage site at arginine residue 1545 is indicated by *RVV-V*.

Recombinant human Factor V and deletion mutants. The recombinant human Factor V (rHFV) used in this study was obtained from a stably transfected Chinese hamster ovary (CHO) cell line (18). CHO DG44 cells, which lack the enzyme dihydrofolate reductase (DHFR) were cotransfected with the expression constructs pDX rHFV and pSV_2 DHFR by calcium phosphate precipitation. Initial selection was performed in media containing exhaustively dialyzed heat-inactivated fetal bovine serum, and surviving clonal colonies were screened for expression of Factor V antigen by an ELISA. One clone expressing 29 ng/ml rHFV was successively amplified in methotrexate-containing media. At a concentration of 80 μM methotrexate, this CHO cell line constitutively expresses $\sim$ 1.5 μg/ml rHFV that is structurally and functionally similar to purified plasma Factor V (18). The rHFV samples used in this study were collected in serum-free media (αMEM/MCDB 302 containing 5 mg/ml BSA and 2.5 mM $CaCl_2$) and had a specific activity of 0.103 U/μg when activated by RVV-V in the chromogenic functional assay (18).

The recombinant Factor V deletion mutants used in this study were prepared from the Factor V cDNA (16) (Fig. 1). All mutants were transiently expressed in COS cells and collected in serum-free bovine albumin-supplemented conditioned media, as previously described (16). The construction, expression, and structural and functional characterization of the following mutants have been described elsewhere: rHFV des C2, rHFV des C1C2, rHFV des LC, rHFV des A3C1, rHFV HC, and rHFV LC (15). In addition to these six deletion mutants, we

1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; CNBr, cyanogen bromide; DHFR, dihydrofolate reductase; PS, phosphatidylserine; rHFV, recombinant human Factor V; RVV-V, Factor V activator from Russell's viper venom.

have also constructed two additional mutants that were used in this study. The mutant rHFV A3, which lacks amino acids 1–1545 and 1878–2196, was constructed by ligating the 5′-noncoding region and the signal sequence of the Factor V cDNA to the cDNA encoding for the third A-type domain. The polymerase chain reaction (PCR) was used with the Factor V cDNA as the template and the oligonucleotides 5′-CACAATCGATGTCGACTCCGGGCTGTCCCAGCTCG-3′ and 5′-ACACGGTACCACTGTGCCGCTTCTGTCCCTT-3′ as primers to produce a fragment containing nucleotides 1–167 of Factor V with a 5′ClaI site and a 3′KpnI site. After restriction with ClaI/KpnI, this fragment was ligated to a KpnI/HindIII restriction fragment from rHFV des C1C2, containing nucleotides 4785–5792 and a TAG stop codon, and a ClaI/HindIII restriction fragment of pBluescript. After DNA sequence analysis of the PCR fragment, the construct was released from pBluescript with SalI and subcloned into the SalI site of pDX, as previously described (16). The mutant rHFV C2, which lacks amino acids 1–2036, was constructed with a BglII/SalI restriction fragment of pDX, containing all of the expression vector except for ~ 100 bp, a BglII/KpnI restriction fragment of pDX rHFV LC containing the remaining nucleotides of pDX and nucleotides 1–167 of Factor V, and a KpnI/SalI restriction fragment of pDX rHFV des A3C1, containing nucleotides 6270–6903 of Factor V. As with the other deletion mutants, these were transiently expressed in COS cells and characterized by immunoprecipitation analysis and for Factor V procoagulant activity, as previously described (15, 16). None of these Factor V deletion mutants possessed procoagulant activity comparable to plasma or recombinant Factor Va.

ELISA analysis. Conditioned media containing rHFV and deletion mutants were analyzed using the biotinylated immunoaffinity-purified rabbit polyclonal anti-human Factor V antibody, as previously described (16). To epitope map the inhibitor, an ELISA microtiter plate (Costar EIA, Cambridge, MA) was coated with a 1:250 dilution of total patient IgG overnight at 4°C. The plate was subsequently blocked with PBS containing 0.2% Tween-20 and 0.25% (wt/vol) nonfat dry milk (blocking buffer). The wells were then incubated with 50-μl aliquots of conditioned media containing rHFV or the individual deletion mutants. Bound Factor V (or deletion mutant) was detected using the rabbit polyclonal anti-human Factor V antibody (16).

Immunoprecipitation of recombinant Factor V and Factor V mutants. Immunoprecipitation analysis of [^{35}S]methionine metabolically labeled recombinant Factor V deletion mutants was performed as previously described (16). For epitope mapping of the inhibitor, patient IgG (1:500 dilution) was substituted for the polyclonal rabbit anti-human Factor V in the immunoprecipitation step with Protein A Sepharose.

Factor V activity assays. Factor Va procoagulant activity was measured using a chromogenic assay, as previously described (16). Briefly, the Factor V sample was first activated with RVV-V (3.75 μg/ml) and added to 50 mM Tris HCl, pH 7.9, 175 mM NaCl, 5 mg BSA/ml (Buffer A) containing 8.0% (vol/vol) rabbit brain cephalin, 0.20 μM prothrombin, and 8.0 mM $CaCl_2$ (final vol: 225 μl). The reaction was initiated by the addition of 25 μl of a 0.1 nM Factor Xa solution and then stopped after 2 min by the addition of 250 μl of buffer A containing 20 mM EDTA. Aliquots of the reaction mixture were transferred to microtiter plate wells, and the amount of thrombin generated during the reaction was determined by the addition of 0.2 mM S2238 (D-Phe-L-pipecolyl-Arg-*p*-nitroanilide) and measuring the change in absorbance at 405 nm using a V_{max} microtiter plate reader (Molecular Devices, Inc., Menlo Park, CA). Standard curves were prepared using purified thrombin.

The effect of the inhibitor on the procoagulant activity of Factor Va was characterized by several approaches. To determine if the inhibitor was time-dependent, 50 μl of rHFV (1.5 μg/ml) in conditioned media was activated with RVV-V and diluted 1:3 into buffer A containing 5 mM $CaCl_2$. The sample was then divided into two 50-μl aliquots, and 4 μl of an 11-μg/ml sample of patient IgG was added to one tube. The reaction mixtures were incubated at 37°C and samples (5 μl) were then removed at various times (0–60 min) and assayed for residual procoagulant activity, as described above. To determine if the inhibitor was temperature-dependent, similar experiments were performed at 22°C. To estimate the relative amount of inhibitor in our IgG sample, 100 μl of rHFV (1.5 μg/ml) in conditioned media was activated with RVV-V, diluted 1:5 into buffer A containing 5 mM $CaCl_2$, and individual 20-μl aliquots were preincubated with 2 μl of various dilutions of patient IgG (or Fab) diluted in Buffer A for 5 min at 22°C before determination of residual procoagulant activity. To determine if preincubation of Factor Va with phospholipids was capable of protecting the activated cofactor from the inhibitor, we incubated an aliquot of Factor Va (20 μl of 0.3 μg/ml RVV-V–activated rHFVa) with 10 μl of rabbit brain cephalin for 5 min at 22°C before addition of the inhibitor (0.15–36.6 μg/ml). The mixture was then incubated an additional 5 min at 22°C before determining residual procoagulant activity. Final concentration of the rabbit brain cephalin was adjusted to 8.0% (vol/vol) in the prothrombinase reaction mixture. Similar experiments were performed to determine the protective effect of preincubation with Factor Xa (0.07 nM) or prothrombin (0.14 μM). For those reactions involving preincubation with Factor Xa, the prothrombinase reaction was initiated by the addition of the prothrombin rather than the Factor Xa.

Subtype analysis. Subtype specific monoclonal antibodies (10 μg/ml in 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) were coated overnight onto microtiter plate wells (Costar EIA). The coating antibodies were removed and the wells were blocked with blocking buffer for 1 h at 37°C. After washing, the wells were incubated for 1 h at 37°C with patient plasma or pooled normal plasma. The wells were then washed and incubated with recombinant Factor V diluted in blocking buffer. Binding of Factor V by the immobilized antibodies was detected using the biotinylated rabbit polyclonal anti-human Factor V antibody (15).

In addition to examining the subtype by ELISA, we also coupled the monoclonal subclass antibodies for anti-human IgG, IgG_1, and IgG_4 to CNBr-activated Sepharose 4B using the manufacturer's recommended procedure. To determine which subclass specific antibody could functionally clear the inhibitor, 40 μl of a 1:20 dilution of the patient IgG fraction in Buffer A was incubated with 10 μl of the antibody-coupled beads for 2 h at 4°C. The beads were removed by centrifugation, and the supernatants were tested for residual inhibitory activity in the chromogenic assay (0.9 nM RVV-V rHFVa incubated with ~ 13.4 nM nonbinding patient IgG for 5 min at 22°C before assay). The beads were then washed and the bound IgG eluted with 0.1 M glycine, pH 2.5. Following removal of the beads by centrifugation, the pH was neutralized with one-tenth vol 2.0 M Tris-HCl, pH 8.0, and assayed for inhibitory activity.

Phospholipid binding assay. The solid-phase ELISA used to detect the binding of Factor V and the deletion mutants to phospholipids has been previously described (15). Briefly, phosphatidylserine (PS) or phosphatidylcholine (PC) dissolved in methanol (3 μg/ml) were air-dried in the wells of a microtiter plate. The wells were then blocked with 50 mM Tris-HCl, 150 mM NaCl, pH 7.2, containing 5 mg/ml gelatin, for 1 h at 37°C, washed, and then incubated with 50 μl aliquots of conditioned media containing recombinant Factor V or a specific deletion mutant. Detection was performed using the biotinylated immunoaffinity-purified polyclonal rabbit anti-human Factor V antibody (15). For experiments determining the effect of the inhibitor and Fab fragments on PS-specific binding of Factor V (or mutants), the conditioned media was incubated with different dilutions of the inhibitor for 5 min at 37°C before incubation in the phospholipid-coated wells.

Results

Inhibition of procoagulant activity. This inhibitor was first detected by the development of prolonged clotting times that did not correct with mixing studies (11). Although Factor VIII inhibitors are reported to be time-dependent, this inhibitor was reported to neutralize the procoagulant activity of Factor V essentially immediately, with no time dependence in mixing studies (11). To optimize incubation times in subsequent experiments, we performed a time-course of inhibition. Using a

fourfold molar excess of patient IgG to Factor Va, the inhibitor completely neutralized the procoagulant activity of Factor Va within 5 min (data not shown). Similar results were obtained with plasma as with recombinant Factor Va, confirming that the recombinant Factor V is very similar to purified plasma Factor V. Control IgG had no effect on the procoagulant activity of rHFVa, nor did the inhibitor block the activity of purified thrombin toward the chromogenic substrate S2238 (data not shown).

This inhibitor was previously reported to comprise ~ 50% of the patient's total IgG (13). When we investigated the effect of dilution on inhibitory activity, we found that a twofold molar excess of patient IgG to rHFVa resulted in < 50% residual procoagulant activity, and a fourfold molar excess resulted in < 20% activity (Fig. 2). When the molar ratio of inhibitor to Factor V was further increased to > 10-fold, the procoagulant activity of Factor Va was completely neutralized, since the reaction proceeded at approximately the same rate in the absence of Factor Va.

To determine if the inhibitory effect of the antibody was secondary to the specific structural epitopes recognized or to steric hindrance from the Fc portion of the immunoglobulin, we prepared and isolated Fab fragments from the inhibitor. These Fab fragments produced a similar inhibitory pattern on dilution (Fig. 2). Furthermore, the inhibitory curve was shifted to the right by a factor of two, consistent with the fact that IgG is a bivalent molecule, whereas the Fab fragment is univalent. As with IgG from normal pooled plasma, papain-generated Fab fragments from normal pooled plasma IgG were noninhibitory at 1,000-fold molar excess (data not shown).

Subtype analysis. The inhibitor was originally reported to be an IgG_4 subclass (12). During the course of our experiments, however, we obtained data that was inconsistant with this interpretation. We therefore reevaluated the subclass of this inhibitor using two different methods and confirmed these results using plasma obtained directly from the laboratory that originally described the inhibitor. When we coated microtiter plate wells with subclass-specific murine anti-human IgGs, we found that the Factor V–binding fraction was an IgG_1 heavy chain subclass (Fig. 3 *a*). Furthermore, murine anti-human

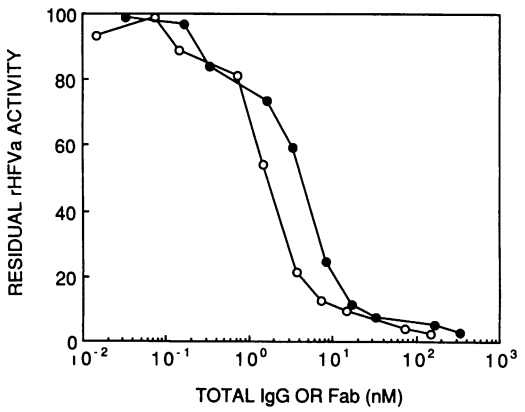

Figure 2. Inhibition of Factor Va procoagulant activity by inhibitor IgG and papain-generated Fab. Recombinant Factor V (~ 0.9 nM) was activated with RVV-V for 3 min at 37°C, and then incubated with increasing dilutions of the inhibitor IgG (○ - ○) or Fab (● - ●) for 5 min at 22°C. Residual Factor Va procoagulant activity was then determined using the chromogenic prothrombinase assay.

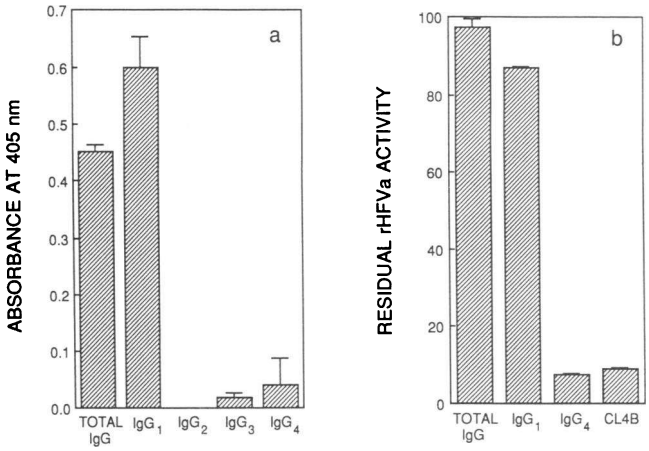

Figure 3. Inhibitor IgG subtype analysis. The inhibitor subtype was determined by two separate methods. In (*a*), microtiter plate wells were coated with murine subtype-specific anti-human IgG antibodies and, after blocking, were incubated with patient plasma. The wells were then incubated with recombinant Factor V, and binding of Factor V was detected using the biotinylated polyclonal anti-human Factor V. This ELISA was performed twice, using two separate murine anti-human IgG_4 clones. In (*b*), murine subtype-specific anti-human IgG antibodies were coupled to CNBr-activated Sepharose CL4B beads and incubated with aliquots of patient IgG. The beads were removed by centrifugation, and the supernatant was assayed for residual Factor Va inhibitory activity in the prothrombinase chromogenic assay, as described in the legend to Fig. 2. The CL4B column represents control glycine-blocked CNBr-activated Sepharose CL4B beads.

IgG_1 antibodies coupled to Sepharose CL4B beads were capable of clearing the inhibitory activity from the patient IgG fraction, confirming that the IgG_1 heavy chain subclass contained the Factor V inhibitor (Fig. 3 *b*). When bound antibody was eluted from the beads, the eluent from the coupled anti-IgG_1 beads possessed Factor Va neutralizing activity (data not shown). Murine anti-IgG_4 subclass antibodies neither bound Factor V in the ELISA, nor did they clear the neutralizing activity from the patient IgG fraction (Fig. 3).

The inhibitor was previously reported to be a monoclonal IgG paraprotein, as determined by preparative isoelectric focusing, but independent studies have reported conflicting results, with one study describing the paraprotein as an IgGλ and another study as an IgGκ. The heavy chain subclass of the paraprotein has not been directly determined. We reexamined the patient's paraprotein by serum protein electrophoresis and confirmed the presence of a small monoclonal component that comprised ~ 30% of the total gamma region (data not shown). Immunofixation analysis revealed two monoclonal components including IgGλ and IgMλ, as well as a possible IgMκ component (data not shown). The observed IgM paraproteins were unrelated to the Factor V inhibitor, since all of the inhibitor bound to Protein A Sepharose. The low ratio of IgG to Factor V that fully inhibits Factor V function suggests that the IgG paraprotein represents the inhibitor. However, the limited amounts of patient plasma available to us were not sufficient to define the gamma chain subclass of paraprotein or to directly demonstrate binding of the paraprotein to Factor V.

Epitope mapping of the Factor V inhibitor. Previous studies with this inhibitor revealed that it bound to the light chain of

Factor Va (14, 19). We have further localized the epitope(s) recognized by this inhibitor by using a series of recombinant light chain deletion mutants of Factor V (Fig. 1). When microtiter plate wells were coated with patient IgG, the only Factor V constructs that bound significantly were rHFV, rHFV des A3C1, and rHFV LC (Fig. 4). Conversely, those constructs that lacked the second C-type domain (rHFV des C2, rHFV des C1C2, and rHFV des LC) did not bind (Fig. 4). The background absorbance was higher than we normally observe in our ELISA, which was caused by residual patient Factor V bound to the Protein A Sepharose purified inhibitor (data not shown). Therefore, to confirm the ELISA, we also used the inhibitor to immunoprecipitate [^{35}S]methionine metabolically labeled recombinant deletion mutants (Fig. 5). As with the ELISA, this confirmed that the inhibitor specifically recognized the C2 domain of Factor V, since mutant constructs lacking the C2 domain were not immunoprecipitated by the inhibitor. Furthermore, the presence of the C1 domain was not necessary for recognition of the C2 domain by the inhibitor, since the individual C2 domain as well as the C2 domain linked to the B domain (from thrombin-activated rHFVa des A3C1) were immunoprecipitated by the inhibitor (Fig. 5, lanes *12* and *13*). Consistent with these findings the Factor V heavy chain component of rHFVa des A3C1 ($\sim$ 105 kD) is precipitated by the polyclonal antibody (lane *7*) but not by the inhibitor (lane *13*).

Inhibition of phosphatidylserine-specific binding. Although the patient plasma samples studied possessed no measurable Factor V procoagulant activity, we did find antigenic Factor V by ELISA at a concentration approximately half that in pooled human plasma (data not shown). This indicates that the inhibitor is a neutralizing antibody and that the Factor V present in patient plasma is in a functionally neutralized immune complex with the inhibitor. When we investigated whether the Factor V in patient plasma was capable of binding to immobilized phosphatidylserine, however, we found that phosphatidylserine-specific binding was significantly decreased in comparison to binding to the polyclonal anti-Factor V antibodies. Almost 90% of the antigenic Factor V in a 1:100 dilution of pooled human plasma bound to the immobilized phosphatidylserine, while < 20% of the antigenic Factor V in a similar dilution of patient plasma was capable of binding to phosphatidylserine. This observation suggested that inhibition of PS-specific binding of Factor V may, at least in part, result in the functional neutralization observed in vivo and in vitro.

We have previously shown that removal of the second C-type domain of Factor V results in the loss of procoagulant activity and phosphatidylserine-specific binding (15). To investigate the effect of the inhibitor on PS-specific binding of Factor V, we preincubated the inhibitor IgG or Fab with rHFV before a phospholipid binding assay. Similar to the inhibition of procoagulant activity, the inhibitor decreased PS-specific binding of Factor V by $\sim$ 40% at a twofold molar excess of inhibitor to rHFV (Fig. 6). The Fab fragments produced a similar inhibitory curve, again shifted to the right similar to the inhibition of procoagulant activity (Fig. 6). In contrast to the inhibitor's effects on procoagulant activity, however, neither the intact inhibitor nor the Fab fragments were capable of completely inhibiting the binding of Factor V to immobilized PS. Even at a greater than 100-fold molar excess of inhibitor or Fab to Factor V, there was still $\sim$ 20–25% residual binding of Factor V to PS (Fig. 6). Conversely, when we first incubated the

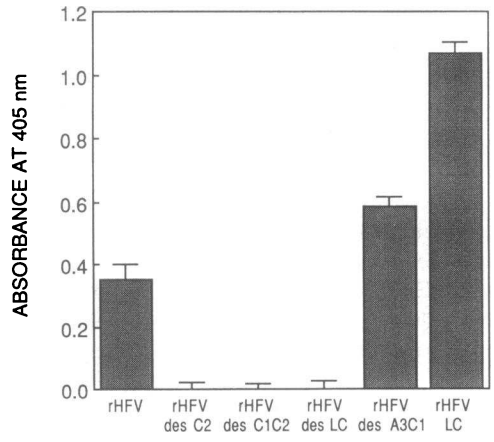

Figure 4. Epitope mapping of inhibitor by ELISA analysis. Microtiter plate wells were coated overnight with the inhibitor IgG (1:250 dilution into PBS). The plates were then blocked and then incubated with the following deletion mutants: rHFV (88 ng/ml), rHFV des C2 (98 ng/ml), rHFV des C1C2 (107 ng/ml), rHFV des LC (94 ng/ml), rHFV des A3C1 (118 ng/ml), rHFV LC (83 ng/ml), and conditioned media from mock-transfected COS cells. The plates were then washed and the same biotinylated polyclonal antibody that was used to determine the concentrations was used to detect binding to the inhibitor. Background absorbance from the mock-transfected conditioned media was 0.55 absorbance units, which was subtracted from the total absorbance of each mutant to determine specific binding.

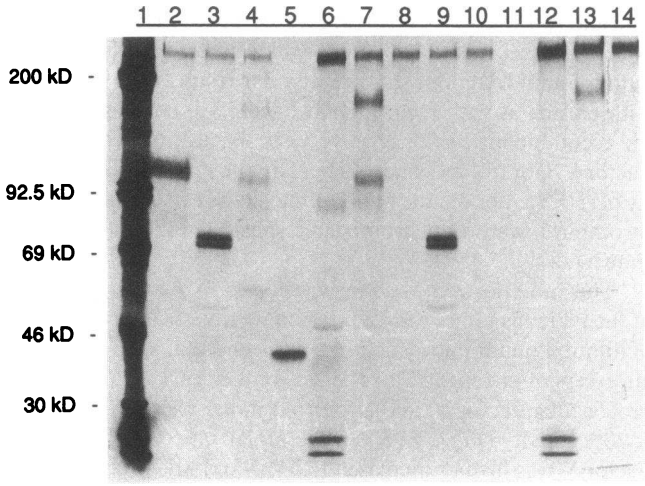

Figure 5. Epitope mapping of inhibitor by immunoprecipitation analysis. Recombinant Factor V deletion mutants metabolically labeled with [^{35}S]methionine were prepared as previously described, and were immunoprecipitated with either the polyclonal anti-human Factor V antibody (lanes *2–7*) or the inhibitor IgG (lanes *8–13*). The precipitated proteins were then electrophoresed on 5–10% gradient SDS-polyacrylamide gels and visualized by autoradiography. The Factor V samples immunoprecipitated were lanes *2* and *8*, rHFV HC; lanes *3* and *9*, rHFV LC; lanes *4* and *10*, thrombin-activated rHFV des C2; lanes *5* and *11*, rHFV A3; lanes *6* and *12*, rHFV C2; lanes *7* and *13*, thrombin-activated rHFV des A3C1. Lane *1* contains the molecular weight standards, and lane *14* contains the control rabbit IgG immunoprecipitate, which contains only the 270-kD contaminant seen in variable amounts in all of our conditioned media samples from COS cells (16).

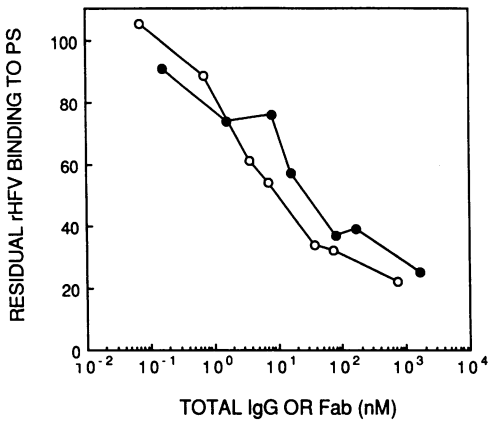

Figure 6. Inhibition of phosphatidylserine-specific binding of Factor V by inhibitor IgG and papain-generated Fab. Recombinant Factor V (~ 1.5 nM) was preincubated with increasing dilutions of inhibitor IgG (○ - ○) or Fab (● - ●) for 5 min at 22°C before incubation with phosphatidylserine-coated microtiter plate wells. Residual bound Factor V was detected using the biotinylated polyclonal anti-human Factor V antibody and alkaline phosphatase streptavidin.

Factor V with immobilized phosphatidylserine for 1 h and then exposed the bound Factor V to a ~ 500-fold molar excess of inhibitor, there was no decrease in the amount of bound Factor V (data not shown). This suggested that once Factor V was found to anionic phospholipids, at least in the phospholipid binding assay, it could not be dissociated by the inhibitor.

The inhibitor and/or Fab fragments were also capable of blocking binding of rHFVa, rHFV des A3C1, and rHFV LC to immobilized phosphatidylserine (data not shown). These results confirmed that the inhibitor was interfering with phosphatidylserine-specific binding mediated by the C2 domain. In contrast, neither total IgG nor papain-generated Fab fragments from pooled human plasma inhibited the phosphatidylserine-specific binding of recombinant Factor V (data not shown).

Protective effect of phospholipids on Factor Va activity. Since the preceeding results suggested that the inhibitor may be interfering with the procoagulant activity of Factor V by blocking PS-specific binding, we investigated whether preincubation of Factor Va with phospholipids would protect the cofactor from neutralization by the inhibitor. When Factor Va was preincubated with rabbit brain cephalin before exposure to the inhibitor, the procoagulant activity was protected from neutralization (Fig. 7). In fact, a > 10-fold increase in the molar ratio of inhibitor to Factor Va was necessary to obtain a comparable level of functional neutralization. A similar protective effect was observed when Factor Va was preincubated with rabbit brain cephalin before exposure to the Fab fragment. In contrast, preincubation with either prothrombin or Factor Xa alone, at the concentrations used in the prothrombinase assay, did not protect Factor Va from the neutralizing effect of the inhibitor (data not shown).

Discussion

Coagulation Factors V and VIII are circulating procofactors that interact with their respective enzyme complexes to localize and amplify a procoagulant response to endothelial injury (6, 20). Factor VIIIa interacts with the enzyme Factor IXa in the presence of calcium ions and a phospholipid membrane or platelet surface to accelerate the activation of the Factor X to Factor Xa. Factor Va interacts with the enzyme Factor Xa in the presence of calcium ions and a phospholipid membrane or platelet surface to accelerate the activation of prothrombin to thrombin. Both cofactors are essential for normal hemostasis, and an absence of either results in a bleeding diathesis. This absence may be the result of a congenital deficiency of the cofactor or the spontaneous development of an autoantibody in a patient with no previous deficiency state. Since inhibitors produce a clinical deficiency state by interfering with the procoagulant activity of a specific factor, the study of coagulation factor inhibitors may provide useful insights into structure-function relationships.

Factor VIII inhibitors have been extensively studied and many have been epitope-mapped to sites within the heavy or light chain of the cofactor (21). Inhibitors that bind to the heavy chain have been localized to the amino-terminal of the A2 domain (21), or to epitopes between the A1 and A2 domains (21, 22). Inhibitors that bind to the light chain have been predominantly localized to the C2 domain of Factor VIII, although some light chain inhibitors that bind to the A3 and/or C1 domains have also been described (21). In contrast to inhibitors that bind to the heavy chain, inhibitors that recognize light chain epitopes (specifically, the C2 domain) interfere with phosphatidylserine-specific binding of Factor VIII (23). Furthermore, preincubation of Factor VIII with phospholipids has been shown to prevent the neutralization of Factor VIII procoagulant activity by inhibitors directed against the light chain (23, 24). Presumably, therefore, these inhibitors disrupt the binding of Factor VIII to a phospholipid membrane or platelet surface, and thereby interfere with the formation of Factor Xa by the "Factor X-ase complex."

In contrast to Factor VIII inhibitors, most Factor V inhibitors have not been characterized beyond the initial studies necessary to establish a diagnosis. Chiu et al. (25) described a neutralizing antibody that spontaneously developed in an 82-yr-old woman. This inhibitor was shown to bind to the heavy chain of Factor Va, and binding of Factor Va by the alkaline

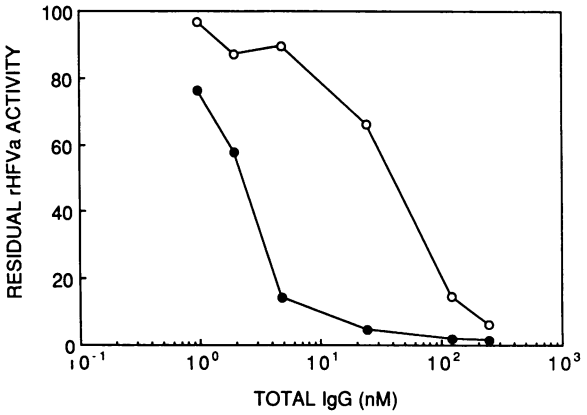

Figure 7. Protective effect of phospholipids on Factor Va procoagulant activity. Recombinant Factor V (~ 0.9 nM) was activated with RVV-V for 3 min at 37°C, and then pre-incubated with rabbit brain cephalin (○ - ○) or buffer A without phospholipids (● - ●) for 5 min at 22°C. The samples were then incubated with increasing dilutions of inhibitor IgG for 5 min at 22°C, and then residual Factor Va activity determined in the prothrombinase chromogenic assay.

phosphatase conjugated inhibitor was blocked by Factor Xa, suggesting that this inhibitor may interfere with the interaction between Factor Va and Factor Xa (25). Zehnder and Leung (4) described a 65-yr-old man who developed antibodies to bovine thrombin and bovine and human Factor V after exposure to a topical thrombin preparation during a porcine mitral valve heterograft. Immunoblot analysis revealed the inhibitor bound to the light chain of Factor V (4), but no studies were performed to elucidate how the inhibitor may be interfering with the procoagulant activity of Factor Va. Lastly, the Factor V inhibitor that forms the basis of this report developed in a 51-yr-old man ~ 10 d after an exploratory laparotomy (11). This antibody was also shown to bind to the light chain of Factor Va (14, 19), and Annamalai et al. (14) observed that the binding of alkaline-phosphatase conjugated inhibitor to immobilized Factor Va was blocked by Factor Xa, suggesting the presence of a Factor Xa binding site in the light chain. Furthermore, this inhibitor was shown to interfere with the Factor Va-mediated binding of Factor Xa to the platelet surface, although whether this was caused by interference with the Factor Va–platelet interaction or the Factor Va–Factor Xa interaction was not determined (13). We did not observe a protective effect of Factor Xa in our prothrombinase assay, however, although this most likely represents differences in the assays used (see below).

Although this antibody was initially reported to be an IgG_4 subtype (12), similar to many Factor VIII inhibitors, we found the factor inhibitor to be an IgG_1 subtype in the two samples we studied. We tested two separate monoclonal anti-IgG_4 antibodies, both of which reacted with myeloma proteins of the appropriate IgG subtype, but neither bound the inhibitor in an ELISA, nor did the anti-IgG_4 antibody clear the Factor V inhibitor from solution. Factor VIII inhibitors within an individual patient may develop a new subtype, reflecting expansion of a new clone from an oligoclonal population of antibodies or possible class switch of the inhibitor (26). This patient's course was relatively brief, however, and we do not know the precise timing when the individual samples were obtained.

Our data indicates that this antibody binds to the second C-type domain of Factor V and effectively neutralizes its procoagulant activity. The inhibitor is capable of binding to the intact (unactivated) molecule as well as the isolated domain, suggesting that the epitope is exposed before procofactor activation. Furthermore, the inhibitor IgG as well as papain-generated Fab fragments interfere with phosphatidylserine-specific binding, and rabbit brain cephalin is able to protect the activated cofactor from neutralization by the inhibitor, suggesting that the inhibitor and procoagulant phospholipids bind at overlapping or spatially adjacent sites. These results confirm and extend our previous studies, which demonstrated that the C2 domain of the Factor V light chain was capable of mediating the specific binding of Factor V to immobilized phosphatidylserine (15). Factor VIII inhibitors that bind to the C2 domain of Factor VIII also block binding of Factor VIII to immobilized phosphatidylserine (23). Taken together, these observations suggest novel strategies for both the treatment of Factor V and Factor VIII inhibitors, as well as the development of new anticoagulant agents that specifically interfere with cofactor function.

The fact that this inhibitor did not completely block binding of Factor V to immobilized phosphatidylserine at concentrations that completely neutralized procoagulant activity suggests that there may be subtle differences in the binding of Factor V to immobilized phosphatidylserine compared to rabbit brain cephalin in solution. The binding of Factor V to phospholipid vesicles and platelets most likely involves more than the C2-mediated interaction with phosphatidylserine alone, since Mann et al. have identified and characterized a region within the third A-type domain of the light chain of bovine Factor V that binds to phosphatidylserine-containing phospholipid vesicles (27, 28). Furthermore, Factor V and Factor VIII do not appear to compete for binding to the platelet surface (29), suggesting that more than anionic phospholipids are required for specific cofactor binding. Alternatively, it is possible that the inhibitor may also be disrupting other interactions in addition to the Factor V–phospholipid interaction. The factor Va light chain appears to contain at least a portion of the high affinity binding sites for Factor Xa and calcium (25, 30). We did not observe a protective effect when Factor Va was preincubated with Factor Xa prior to exposure to the inhibitor in the chromogenic prothrombinase assay, however. The absence of a protective effect under these conditions may be a reflection of the significantly weaker association between Factor Xa and Factor Va in the absence of phospholipids (31). Consistent with this weaker interaction, the concentrations of Factor Xa that were reported by Annamalai et al. (14) to block binding of inhibitor to immobilized Factor Va were several hundredfold higher than those employed in our study. We did observe that preincubation of Factor Va with rabbit brain cephalin and Factor Xa results in an increased protective effect compared to preincubation with rabbit brain cephalin alone (Ortel, T. L., and W. H. Kane, unpublished observations), although whether this was caused by the Factor Va–Factor Xa interaction or the enhanced affinity of Factor Va for a membrane surface in the presence of Factor Xa is unknown. Experiments are currently in progress using synthetic peptides, monoclonal antibodies, and additional recombinant constructs to further dissect those regions in the light chain that mediate inhibitor binding, anionic phospholipid binding, Factor Xa binding, and procoagulant activity.

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