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Catalytic IgG from Patients with Hemophilia A Inactivate Therapeutic Factor VIII[1]

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Factor VIII (FVIII) inhibitors are anti-FVIII IgG that arise in up to 50% of the patients with hemophilia A, upon therapeutic administration of exogenous FVIII. Factor VIII inhibitors neutralize the activity of the administered FVIII by sterically hindering its interaction with molecules of the coagulation cascade, or by forming immune complexes with FVIII and accelerating its clearance from the circulation. We have shown previously that a subset of anti-factor VIII IgG hydrolyzes FVIII. FVIII-hydrolyzing IgG are detected in over 50% of inhibitor-positive patients with severe hemophilia A, and are not found in inhibitor-negative patients. Although human proficient catalytic Abs have been described in a number of inflammatory and autoimmune disorders, their pathological relevance remains elusive. We demonstrate here that the kinetics of FVIII degradation by FVIII-hydrolyzing IgG are compatible with a pathogenic role for IgG catalysts. We also report that FVIII-hydrolyzing IgG from each patient exhibit multiple cleavage sites on FVIII and that, while the specificity of cleavage varies from one patient to another, catalytic IgG preferentially hydrolyze peptide bonds containing basic amino acids. ***The Journal of Immunology,*** **2006, 177: 1355–1363.**

Hemophilia A is an inherited X-linked bleeding disorder that is characterized by the absence of functional procoagulant factor VIII (FVIII)[3] in the circulation (1). Treatment of hemophilia A with therapeutic administration of FVIII results in the generation of anti-FVIII Abs of the IgG isotype that inhibit FVIII activity (FVIII inhibitors) in ~50% of the patients (2). The occurrence of FVIII inhibitors appears as the major complication of hemophilia A treatment. Factor VIII inhibitors neutralize the activity of FVIII by preventing its interaction with other molecules of the coagulation cascade by steric hindrance (3–8), or by forming immune complexes that accelerate FVIII clearance from the circulation (9). In addition to these mechanisms, we have demonstrated the presence of Abs that hydrolyze FVIII in inhibitor-positive patients with severe hemophilia A (10). FVIII-hydrolyzing Abs were found in >50% of inhibitor-positive hemophilic patients, and were not detected in inhibitor-negative patients (11). The rates of FVIII hydrolysis were found to correlate with the inhibitory activities scored in the plasma of the patients (11).

Human proficient catalytic Abs have been described in the context of inflammatory and autoimmune disorders, including asthma, Hashimoto's thyroiditis, multiple myeloma, systemic lupus erythematosus, scleroderma, rheumatoid arthritis, multiple sclerosis, and HIV-related immune thrombocytopenia (12–18). Although there is evidence supporting a detrimental role for a subset of platelet-fragmenting Abs in HIV infection (18), the deleterious role of catalytic Abs in the other disorders remains debated. In this study, we demonstrate in vitro, in the presence of human serum albumin (HSA), that the kinetics of FVIII degradation by FVIII-hydrolyzing IgG are compatible with a pathogenic role for catalytic Abs in inactivation of the therapeutic FVIII administered to hemophilia A patients. We also report that FVIII-hydrolyzing IgG from each patient exhibit multiple cleavage sites on FVIII and that, while the specificities of cleavage vary from one patient to another, Ab catalysts preferentially hydrolyze peptide bonds containing basic amino acids. Our findings are critical for the design of generic neutralizing molecules against anti-FVIII IgG catalysts.

Materials and Methods

Patients

Frozen plasma samples of 24 inhibitor-positive patients with severe hemophilia A (plasma FVIII levels <1.0% of normal) were obtained from the following institutions in accordance with the local ethical regulation: Hôpital Cochin (Paris, France), Hôpital du Kremlin-Bicêtre (Bicêtre, France), Hôpital de Saint-Etienne (Saint-Etienne, France), Gasthuisberg Leuven (Leuven, Belgium), and the Christian Medical College Hospital (Vellore, India). Most of the patients had been diagnosed with severe hemophilia A within the first 2 years of life. The mean age of the patients at the time of blood sampling was 40.6 ± 18.4 years (mean ± SD, ranging from 10 to 70). Patients could be divided in three groups according to the inhibitory

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[3] Abbreviations used in this paper: FVIII, factor VIII; IVIg, i.v. Ig; BU, Bethesda unit; PVDF, polyvinylidene difluoride; FIX, factor IX; FX, factor X; HSA, human serum albumin; PFR, proline-phenylalanine-arginine; MCA, methylcoumarinamide.

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activity measured in plasma using the Bethesda assay. Mean inhibitory activities in each group were 2.7 ± 1.5 Bethesda units (BU)/ml (3 low responder patients, ranging from 1 to 4 BU/ml), 8.5 ± 1.4 BU/ml (4 patients with moderate inhibitory titers, 6.7–10.0 BU/ml), 65.5 ± 65.0 BU/ml (17 patients with high inhibitory titers, 12–280), respectively. Plasma from inhibitor-positive patients with mild hemophilia, and from inhibitor-positive patients with severe hemophilia undergoing immune tolerance induction, were not included in our study.

Purification of IgG

IgG was isolated from plasma by 50% ammonium sulfate precipitation followed by affinity-chromatography on protein G-Sepharose (Amersham Biosciences). A therapeutic preparation of pooled normal human IgG (i.v. Ig (IVIg), Sandoglobulin) was used as a source of normal IgG. Size-exclusion chromatography of patients' IgG and IVIg was performed on a Superose-12 column (Pharmacia) equilibrated with 50 mM Tris, 8 M urea, and 0.02% NaN_3 (pH 7.7), at a flow rate of 0.25 ml/min to exclude contaminating proteases. IgG-containing fractions were then pooled and dialyzed against 50 mM Tris, 100 mM glycine, 0.02% NaN_3 (pH 7.7) for 2 days at 4°C. We have previously demonstrated that urea-treated purified IgG retain the inhibitory activity toward FVIII (11). The purity of IgG preparations was confirmed 1) by SDS-PAGE and immunoblotting under nonreducing conditions, 2) by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, and 3) by coincubation of the IgG with a biotinylated suicide inhibitor for serine proteases (phosphonate diester covalently reactive analog; a gift from Prof. S. Paul, University of Texas, Houston, TX) followed by detection in Western blot of the biotin-labeled material. IgG was quantified by ELISA.

Purification of anti-FVIII IgG Abs by affinity chromatography

IgG from patients B1, B2, C1, N1, P1, and Wal reactive to FVIII were affinity purified on a matrix to which human FVIII had been coupled. Two different matrices and coupling protocols were used. Twenty-five thousand units (5 mg) of immunopurified plasma-derived FVIII (LFB) were coupled to 10 ml of cyanogen bromide-activated Sepharose (Pharmacia). IgG of patients B2 and C1 were purified as described previously (19). Briefly, following overnight incubation with the affinity matrix at 4°C and extensive washing with PBS, anti-FVIII IgG were eluted using 0.2 M glycine (pH 2.8), dialyzed against PBS, 0.01% NaN_3 and concentrated with Centriprep (Amicon). Alternatively, 12,000 U (2.4 mg) of human recombinant FVIII (Hyland) were coupled to 6.7 ml of Affigel-HZ (Bio-Rad) through glycosylated moieties of FVIII as previously described (20). Following incubation of IgG from patients B1, N1, and P1 with the FVIII matrix and extensive washing, anti-FVIII IgG were recovered by sequential acid (50 mM imidazole (pH 4.5), 60% ethylene glycol, 0.5% HSA, 40 mM $CaCl_2$), and alkaline elutions (50 mM diethylamine (pH 10), 60% ethylene glycol, 40 mM $CaCl_2$), dialyzed, and concentrated.

Biotinylation of Ags

Recombinant human FVIII (Kogenate II; Bayer) was reconstituted in distilled water to a final concentration of 396 μg/ml, desalted on PD-10 columns (Pharmacia) and eluted in 500-μl fractions using borate buffer (100 mM borate (pH 7.0), 150 mM NaCl, 5 mM $CaCl_2$). Fractions that contained FVIII as assessed by direct ELISA were pooled. Biotin (440 μl at 25 μg/ml) was allowed to react with 3 ml of FVIII at 198 μg/ml with gentle agitation in the dark for 2 h at 4°C. Biotinylated FVIII was dialyzed against borate buffer for 2 h at 4°C, aliquoted, and stored at −20°C until use. The protocol was essentially identical for the biotinylation of human plasma-derived albumin (HSA; LFB) and recombinant human factor IX (FIX; BeneFix; Baxter).

Hydrolysis of biotinylated Ags

Biotinylated FVIII, HSA, and FIX (385 nM) were incubated in 40 μl of catalytic buffer (50 mM Tris-HCl (pH 7.7), 100 mM glycine, 0.025% Tween 20, and 0.02% NaN_3) with the IgG samples (25 μg/ml, 167 nM) to be tested for 12–48 h at 37°C. Samples were mixed with Laemmli buffer without ME (1:1 v/v), and 20 μl of each sample was subjected to 10% SDS-PAGE. Protein fragments were then transferred onto nitrocellulose membrane (Schleicher & Schuell). Following overnight blocking in PBS, 1% BSA, 0.1% Tween 20 at 4°C, membranes were incubated with streptavidin-coupled horseradish-peroxidase (Pharmacia) diluted 1/3000 in blocking buffer, for 30 min at room temperature. After washing in PBS containing 0.1% Tween 20, labeled proteins were revealed using the ECL kit (Amersham Biosciences) and BIOMAX ML films (Kodak). Films were scanned using a SnapScan 600 (Agfa) scanner.

Determination of FVIII inhibitory activity

FVIII inhibitory activity was measured in purified IgG preparations using the Bethesda assay (21). Purified IgG and anti-FVIII IgG were incubated with an equal volume of pooled citrated human plasma (Dade-Behring) for 2 h at 37°C. Residual FVIII activity was measured in a one-stage clotting assay by determination of the activated partial thromboplastin time using human plasma depleted of FVIII (Dade-Behring) as substrate, human placental pathromtin (Behring) as activators and a reference plasma pool (Dade-Behring). Dilutions were conducted in Owren's Veronal buffer (Dade-Behring). Interassay variation ranged between 1 and 2.5%. FVIII inhibitory activity was expressed as BU per milligram of IgG.

Modified Bethesda assay for FVIII-hydrolyzing Abs

IgG from patient Wal, B1, and from the human monoclonal anti-FVIII B cell clone B02C11 (a gift from Prof. J. M. Saint-Remy, Center for Molecular and Vascular Biology, Katolische Universitat Leuven, Leuven, Belgium), were incubated at concentrations yielding 70–80% residual FVIII activity (i.e., 11.2 μg/ml, 1 μg/ml and 15 ng/ml, respectively) with rFVIII (3 IU/ml; Kogenate) in Owren Veronal buffer supplemented with 1 mg/ml HSA, for 2, 4, 8 and 16 h at 37°C. FVIII incubated in Veronal-HSA alone was used as a control. The residual FVIII activity was determined at each time point using a conventional one-stage coagulation assay. IVIg, incubated at 11.2 μg/ml for up to 16 h as a negative control, did not neutralize FVIII activity (data not shown).

Sequencing of cleavage fragments

In the case of patients C1, I1, and P1, unlabeled FVIII (300 μg, Kogenate II) was treated with the purified IgG (37 μg) in 1.5–3.0 ml of catalytic buffer for 42–48 h at 37°C. Alternatively, in the case of patient Wal, FVIII (300 μg) was treated with affinity-purified anti-FVIII IgG (74 μg) in 1500 μl of catalytic buffer for 24 h at 37°C (10). The resultant FVIII cleavage fragments were run on a 10% SDS-PAGE at 50 mA under nonreducing conditions and transferred for 2 h at 100 mA on a Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences) in 10 mM CAPS, 10% ethanol (pH 11.0). After staining with Coomassie blue, visible bands (molecular mass between 27.3 and 94.1 kDa) were cut and subjected to N-terminal sequencing for 10 cycles, using an automatic protein microsequencer Prosize 492 cLC (Applied Biosystems). FVIII incubated in buffer alone did not yield cleavage bands that were detectable upon staining with Coomassie blue. Amounts of protein sequenced >0.5 pM were considered significant and above the background level associated with the experimental conditions. Each visible band in the electrophoretic profiles yielded 2.4 ± 1.2 different sequences (average ± SD, ranging from 1 to 7). Conversely, each individual sequence was detected in 2.8 ± 1.9 electrophoretic bands (ranging from 1 to 8). All sequences were identified as cleavage sites of FVIII.

Hydrolysis of Pro-Phe-Arg-methylcoumarinamide (MCA)

IgG Abs (167 nM) were mixed with proline-phenylalanine-arginine (PFR) MCA (PFR-MCA; Peptide Inc.) at 0–1.7 mM in 40 μl of 50 mM Tris-HCl, 100 mM glycine, 0.025% Tween 20, and 0.02% NaN_3 (pH 7.7) in white 96-well U-bottom plates and incubated in the dark for 12–42 h at 37°C. Hydrolysis of the PFR-MCA substrate was determined by the fluorescence of the leaving group (aminomethylcoumarin; λ_{em} 460 nm, λ_{ex} 370 nm) using a fluoroscan. Fluorescence values were compared with a standard curve of free MCA and the corresponding quantities of released MCA were computed. At each time point, background release of MCA, measured for each PFR-MCA concentration in wells containing the substrate alone, was subtracted from the value observed in the presence of the Abs. Data are expressed as the quantity of released MCA computed at time 0 subtracted from the quantity of released MCA computed at a given time point, per amount of time per amount of IgG. The significance of the increase in the hydrolytic activity of patient IgG as compared with that of IVIg was assessed by an ANOVA and Fischer post hoc tests. The reported *p* values are two-sided.

Results

IgG from inhibitor-positive hemophilia A patients hydrolyze FVIII

We first investigated the capacity of IgG purified from the plasma of inhibitor-positive patients with hemophilia A to hydrolyze human recombinant FVIII. FVIII exhibits a characteristic electrophoretic pattern, with major protein bands migrating at molecular masses between 250 and 337 kDa and an additional band at 82 ±

2 kDa (Fig. 1). Incubation of FVIII with IgG from inhibitor-positive patients C1, F1, and P1 resulted in FVIII hydrolysis and in the generation of up to eight major digestion fragments of molecular masses ranging from 52.5 to 26.4 kDa (Fig. 1). In contrast, the migration profile of FVIII remained unchanged when it was incubated in buffer alone (Ctl), or in the presence of normal polyclonal human IgG (IVIg) (data not shown). IgG from inhibitor-positive patient R2 did not hydrolyze FVIII either. HSA and recombinant human FIX, which present single electrophoretic protein bands of 65 and 56 kDa, respectively, were not hydrolyzed when incubated with IgG of patients C1, F1, P1, and R2, under similar experimental conditions (Fig. 1).

The hydrolysis of FVIII by IgG was studied in the case of 24 inhibitor-positive patients with severe hemophilia A (11). The migration profiles of hydrolyzed FVIII were subjected to densitometric analysis so as to compute the rate of FVIII hydrolysis (Fig. 2, □). IVIg exhibited a marginal hydrolytic activity of 0.04 ± 0.04 pM/min/nM. The latter value was consistently lower than that of the hydrolytic activity of patients' IgG. Purified IgG from 13 of the 24 patients with severe hemophilia (patients B1, B2, B3, B4, C1, C2, F1, H1, I1, N1, P1, V1, and Y1) exhibited a significantly higher FVIII-hydrolyzing activity than IVIg ($p < 0.05$). The mean hydrolytic activity of IgG of these 13 patients was 0.48 ± 0.28 pM/min/nM.

FVIII-hydrolyzing IgG neutralize FVIII procoagulant activity

Using a one-stage coagulation assay, we investigated whether IgG-mediated hydrolysis of FVIII results in FVIII inactivation. We diluted the IgG from two inhibitor-positive patients to concentrations at which "classical" FVIII inhibition by steric hindrance measured in the Bethesda assay, is marginal (75–80% of residual FVIII activity after 2 h of incubation). We also increased the time of incubation up to 16 h. In such conditions, classical FVIII inhibitors are at equilibrium with FVIII and the residual FVIII activity remains constant with time, unless FVIII is hydrolyzed by anti-FVIII IgG, thus inducing a decrease in the residual FVIII activity (Fig. 3*A*). Incubation of FVIII with a human monoclonal FVIII inhibitor devoid of FVIII-hydrolyzing activity, BO2C11, resulted in steady levels of residual FVIII activity during 16 h (i.e., 80.3 ± 5.8%). In contrast, incubation of FVIII with IgG from patients Wal and B1 resulted in a significant time-dependent decrease of residual FVIII activity (Fig. 3*B*; $p < 0.01$ and $p < 0.05$, respectively). Thus, at IgG concentrations much inferior to that found in normal plasma (11.2 and 1 μg/ml for IgG from patients Wal and B1, respectively), half of the FVIII available in the assay was inactivated after 7 and 16 h, respectively, values inferior or equal to the normal half-life of therapeutic FVIII in circulation (22–24). Importantly, the assays were performed in the presence of 1 mg/ml HSA, thus making the experimental conditions close to physiological ones.

Cleavage site specificity of factor VIII-hydrolyzing IgG

Recombinant human FVIII was incubated with IgG of patients C1, I1, and P1, and with affinity-purified anti-FVIII IgG of previously described patient Wal (10). The peptide fragments generated were resolved by 10% SDS-PAGE and Western blotting before being subjected to N-terminal sequencing. The latter approach allowed the identification of scissile bonds located in all domains of FVIII except those in the C2 domain. The 45 identified protein sequences all belonged to the FVIII molecule (Table I), indicating the absence of contamination by adventitious proteins. IgG of patients Wal, P1, C1, and I1 exhibited 7, 17, 13, and 25 different cleavage sites each: six of these sites were common to IgG of three patients (i.e., K36-S37, R336-M337, R740-S741, K974-G975, K992-V993, K1098-M1099, and R1313-A1314), three sites were common to two patients (i.e., K408-S409, K661-M662, and R1422-K1423). The remaining cleavage sites were unique to IgG of individual patients.

The A1A2, B, and A3C1 domains represent 34.1, 41.8, and 24.1% of the size of the FVIII molecule, respectively, excluding the C2 domain. The 45 identified cleavage sites were evenly distributed among the domains (Spearman rank correlation test: $\rho = 0.76$; $r^2 = 0.97$; $p < 0.02$). Thus, ~39% of the 45 sites were located in the A1A2 domains, whereas 47% were located in the B domain and 14% in the A3C1 region, suggesting that catalytic anti-FVIII IgG have no preferential specificity for either the H chain, the L chain, or the B domain of FVIII. The amino acid residues at the 45 scissile bonds differed in charge, size, and hydrophobicity. Thus, the site preceding (N-terminal to) the scissile bond was occupied by K (48.9%), R (35.6%), Y (4.4%), T (2.2%), M (2.2%), E (2.2%), L (2.2%), or F (2.2%), and the site C-terminal to the scissile bond by A (2.2%), D (8.9%), E (6.7%), F (4.4%), G (4.4%), I (2.2%), K (13.3%), M (11.1%), N (4.4%), Q (4.4%), S (22.2%), T (2.2%), or V (13.3%). Together, cleavages occurred in 84.5% after K or R, indicating that proteolytic IgG preferentially behave similar to serine proteases.

Using the Swiss-pdb viewer software and the five-domain model of FVIII that includes amino acids 1–336, 376–716, and 1692–2332 (Table I), we determined that 12 of the 19 cleavage

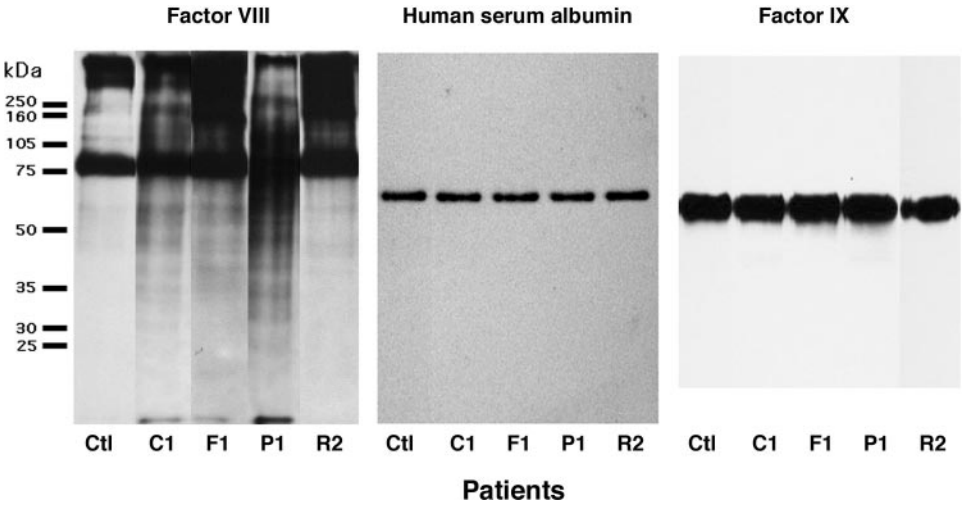

FIGURE 1. FVIII-hydrolyzing activity of IgG purified from plasma of inhibitor-positive patients with severe hemophilia A. Biotinylated FVIII, HSA, and factor IX (385 nM) were incubated alone (Ctl) or in the presence of IgG Ab (167 nM) purified from plasma of patients with severe hemophilia A for 24 h at 37°C. Samples were separated by 10% SDS-PAGE. Results are shown for patients C1, F1, P1, and R2. Molecular masses are indicated in the *left margin*.

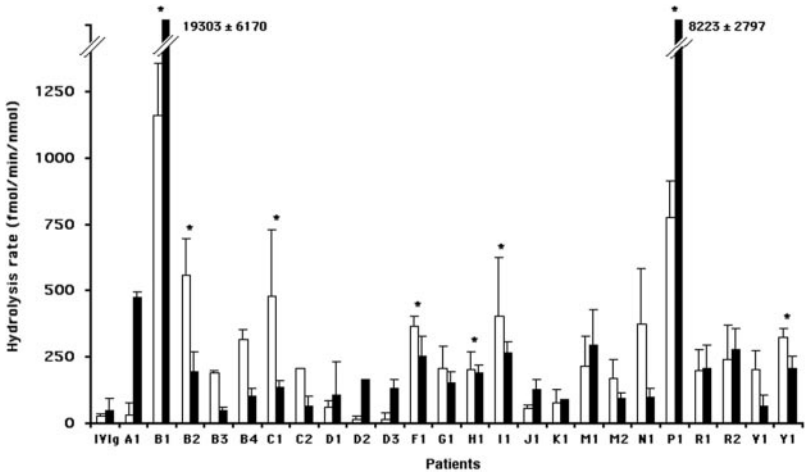

FIGURE 2. Hydrolysis rates of IgG from inhibitor-positive patients with severe hemophilia A. Biotinylated FVIII (385 nM, □) or the MCA-coupled PFR peptide (PFR-MCA, 100 μM, ■) were incubated alone (Ctl) or in the presence of IVIg or of IgG (167 nM) purified from the plasma of 24 patients for 24 h at 37°C. In the case of FVIII hydrolysis, samples were separated by 10% SDS-PAGE, and the rate of hydrolysis was quantified by scanning of immunoblots. Spontaneous hydrolysis occurring upon incubation of FVIII in the presence of buffer alone was considered as background and subtracted. The data represent the means and SDs of femtomoles of FVIII hydrolyzed per minute per nanomole of IgG, for two or three separate experiments. The mean coefficient of variation was 0.33 (range, 0.01–1.41). In the case of hydrolysis of PFR-MCA, the fluorescence of released MCA was measured. Spontaneous hydrolysis occurring upon incubation of PFR-MCA in the presence of buffer alone was considered as background and subtracted. The data represent the means and SDs of three or four separate experiments. The mean coefficient of variation is equal to 0.32 (0.01–1.14). IgG from eight patients exhibited hydrolysis rates that were significantly greater than that of IVIg both for FVIII and PFR-MCA (*, $p < 0.05$, as assessed using the ANOVA post hoc test).

sites available in the model are buried in the molecule (i.e., accessibility ≤20%), while 7 cleavage sites are exposed at the surface of FVIII and are readily accessible for interacting with other molecules. The other IgG cleavage sites on the FVIII molecule, which are not available in the model and which overlap with the natural cleavage sites for thrombin, activated factors IX and X (FIXa and FXa), and activated protein C, i.e., R336-M337, R372-S373, R740-S741, R1313-A1314, and R1648-E1649 (Table II), can also be assumed to be highly exposed. Conversely, only few of the amino acids of FVIII that are exposed at the surface of the molecule, were identified as cleavage sites, thus suggesting that catalytic IgG target specific structures in the FVIII molecule.

IgG from inhibitor-positive patients exhibit hydrolytic activity toward PRF-MCA

Incubation of patients' IgG with the peptide PFR-MCA resulted in hydrolysis of the peptide and release of the fluorescent MCA tag, thus allowing for the calculation of rates of hydrolysis. Using PFR-MCA as a substrate enables the study of the kinetics of cleavage at a single bond (i.e., Arg-MCA), as opposed to the average kinetics observed when measuring the cleavage of multiple bonds in a large substrate such as FVIII.

Hydrolysis of PFR-MCA was dose and time dependent (data not shown). IVIg, used as a negative control, demonstrated a marginal PFR-MCA-hydrolyzing activity of 0.04 ± 0.04 pM/min/nM (Fig. 2, ■). IgG of 13 patients (patients A1, B1, B2, C1, F1, G1, H1, I1, M1, P1, R1, R2, and Y1) displayed significantly higher hydrolytic rates toward PFR-MCA than that observed with IVIg ($p < 0.05$). IgG of the remaining 11 patients exhibited hydrolysis rates of PFR-MCA that were either marginal as compared with IVIg or for which interexperimental differences did not allow to reach significance. The rate of hydrolysis of PFR-MCA by IgG from patients B1 and P1 was 19.30 ± 6.17 and 8.22 ± 2.80 pM/min/nM, respectively, which was at least 20-fold higher than the mean hydrolysis rate of IgG of the other patients (0.24 ± 0.09 pM/min/nM) (Fig. 2). Altogether, IgG of 8 of 24 patients (i.e., patients B1, B2, C1, F1, H1, I1, P1, and Y1) displayed hydrolytic activities significantly greater than that of IVIg in the case of both FVIII and PFR-MCA (Fig. 2). Rates of hydrolysis of FVIII and PFR-MCA for these eight patients were positively correlated (one-tailed Fisher exact test, $p < 0.05$).

FVIII-specific IgG were further purified by affinity chromatography in the case of patients B1, B2, C1, N1, and P1. The yield of the purifications ranged between 50 and 300 μg/10 mg of IgG, representing relative enrichments of 33- to 200-fold. The specific FVIII inhibitory activity of unfractionated IgG and of affinity-purified anti-FVIII IgG were measured by means of the Bethesda assay. Affinity purification of anti-FVIII IgG resulted in a 65-fold mean increase in FVIII inhibitory activity (ranging from 11- to 190-fold; Table III), a value which is in the same range as that of the weight-wise enrichment, demonstrating the specificity for FVIII of the affinity-purified material.

We then monitored the specific hydrolytic activity of unfractionated IgG and of affinity-purified anti-FVIII IgG using PFR-MCA as a substrate. Affinity purification of anti-FVIII IgG resulted in an enrichment in specific hydrolytic activity of PFR ranging between 2- and 315-fold, with a mean increase of 59-fold (Table III). These results are in agreement with our previous observations of an enhancement of hydrolysis of FVIII by affinity-purified FVIII-specific IgG from patients' plasma (10, 11). Importantly, excess of FVIII was able to neutralize IgG-mediated hydrolysis of PFR-MCA (Fig. 4, shown in the case of patient C1). The calculated *Ki* was equal to 0.12 ± 0.01 μM.

Together, our data thus indicate that catalytic anti-FVIII IgG is endowed with a serine protease-like hydrolytic activity and that PFR-MCA may be a suitable alternative substrate for studying the kinetic parameters associated with FVIII-specific proteolysis by patients' IgG.

Kinetics of proteolysis by catalytic Abs of hemophilic patients

The kinetic parameters of hydrolysis by catalytic anti-FVIII IgG were calculated in the case of patients B1, B2, B4, C1, F1, I1, and

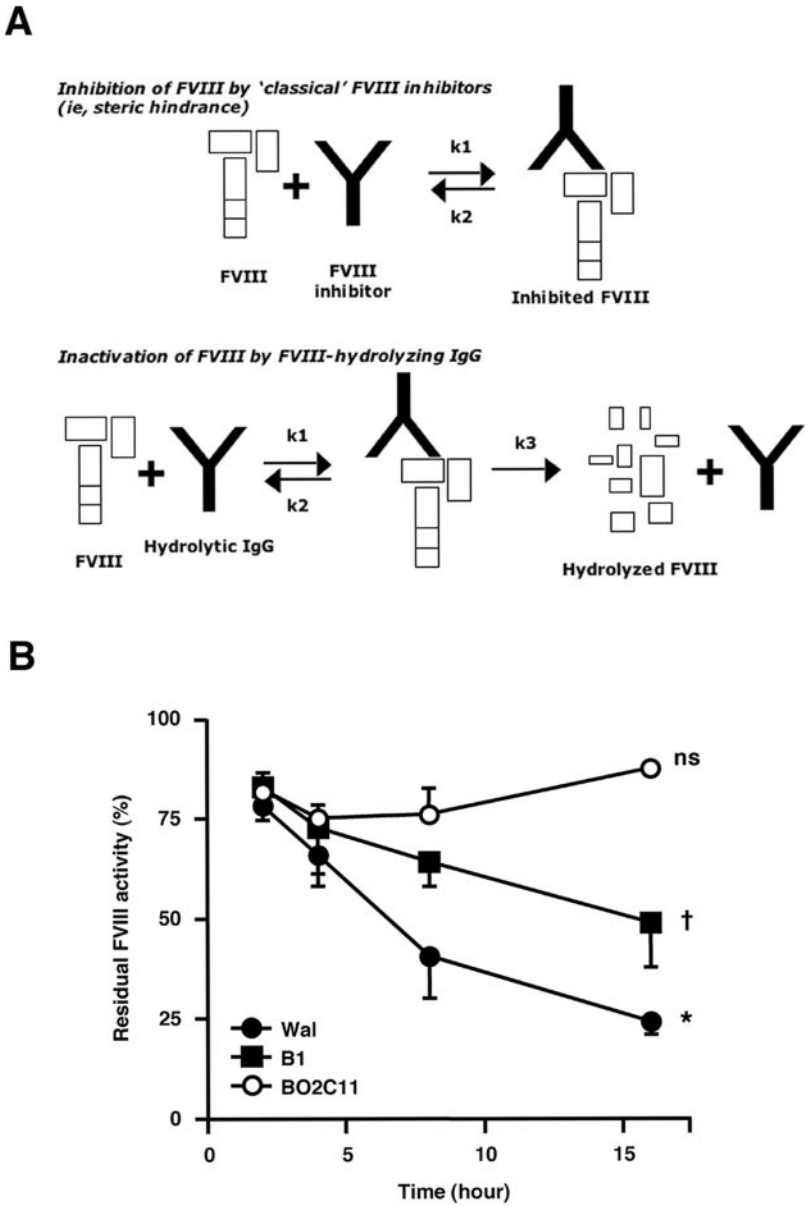

FIGURE 3. FVIII inactivation by FVIII-hydrolyzing IgG. *A*, Classical FVIII inhibitors prevent the interaction of FVIII with other molecules of the coagulation cascade by steric hindrance, and form steady-state interactions with FVIII in in vitro coagulation assays. The amount of FVIII available for coagulation is thus stable once equilibrium is reached, i.e., for an incubation period above 2 h. In contrast, FVIII-hydrolyzing IgG degrade FVIII: the amount of FVIII available for coagulation thus decreases with time. The two mechanisms of FVIII inactivation may coexist in a single patient. *B*, FVIII (3 IU/ml) in Veronal buffer-HSA (1 mg/ml) was incubated alone or in the presence of IgG from patients Wal (11.2 μg/ml) and B1 (1 μg/ml), as well as IgG from the monoclonal anti-FVIII B cell clone BO2C11 (15 ng/ml) for 2, 4, 8, and 16 h at 37°C. Residual FVIII activity was measured at each time point using the Bethesda assay. The figure depicts the mean residual FVIII activity (±SE mean), relative to the residual activity of FVIII incubated in buffer alone, as a function of time, from two to four independent experiments. The time-dependent decrease in residual FVIII activity was significant in the case of IgG from patients Wal (*, $p < 0.01$) and B1 (†, $p < 0.05$), as assessed by a one-way ANOVA (ns, nonsignificant).

P1. In all these cases, incubation of patients' IgG with increasing concentrations of PFR-MCA led to the saturation of the hydrolytic activity toward PFR-MCA (data not shown; $r^2 \geq 0.98$). The curves of the reciprocal of the velocity plotted as a function of the reciprocal of the substrate concentration were linear (data not shown), indicating that the simple Michaelis-Menten kinetics is an appropriate model for the reaction. Fitting the experimental data to the Michaelis-Menten equation allowed the derivation of the apparent V_{max} and average K_m of the reactions (Table IV). The computed kinetic parameters were heterogeneous among patients' IgG. The mean apparent V_{max} ranged from 6.1 to 609.4 fmol/min. The calculated average K_m ranged between 467 ± 125 μM and 1400 ± 1248 μM with a mean value of 794.2 ± 300.8 μM. The nominal K_{cat} values, which represent the sum of the individual constants of anti-FVIII IgG within each polyclonal preparation, were between 0.03 and 2.61 min^{-1} (Table IV). Computed catalytic efficiencies were between 37.0 and 3957.7/M/min, respectively.

Discussion

Anti-FVIII Abs in patients' plasma are a composite mixture of catalytic and noncatalytic Abs. The FVIII-neutralizing activity of FVIII inhibitors in vivo thus results from two simultaneous mechanisms: steric hindrance of the interaction of FVIII with relevant molecules of the coagulation cascade, and proteolysis of FVIII by the subset of Abs with catalytic properties. Although the pathological relevance of the Ab catalysts reported in different autoimmune and inflammatory disorders remains elusive, we demonstrate here that the kinetics of FVIII degradation by FVIII-hydrolyzing IgG are compatible with a pathological role for catalytic Abs.

The activity of FVIII inhibitors is generally assessed using the Bethesda assay (21). Although the Bethesda assay requires up to 2 h to reach complete FVIII inactivation, the incubation of FVIII with purified patients' IgG requires 24 h before hydrolysis is detected, a 12-fold difference. However, in the Bethesda assay, the molar ratio of IgG to FVIII is >200 molecules of IgG for 1 molecule of FVIII (IgG concentration in diluted plasma of a patient with 1000 BU/ml would be 10 μg/ml and FVIII in reference plasma is 100 ng/ml), whereas it is of 0.5 IgG for one FVIII molecule in the hydrolysis assay (IgG and FVIII concentrations are 25 and 100 μg/ml, respectively), a 400-fold difference. Indeed, the accuracy of the hydrolysis assay is subject to technical constraints related to the sensitivity of the detection of digested fragments of

Table I. *Sites of FVIII cleavage by anti-FVIII IgG of hemophilia A patients*[a]

Sequence of FVIII	Patients				3D[b]	Accessibility of Amino Acid[c]
	Wal	P1	C1	I1		
		K36-S37	K36-S37	K36-S37	+	40
				R121-E122	+	30
				K127-V128	+	10
				K166-D167	+	10
		R240-S241			+	10
		K251-S252			+	10
			Y323-V324		+	10
A1–A2 domains		R336-M337	R336-M337	R336-M337	+	40
	R372-S373					
1–740		K408-S409		K408-S409	+	30
				R418-I419	+	10
		R583-S584			+	20
			R593-F594		+	40
	T646-D647				+	20
		K661-M662		K661-M662	+	20
			F679-M680		+	20
				R698-N699	+	20
B domain		R740-S741	R740-S741	R740-S741		
741–1648				R752-Q753		
				R776-T777		
		K872-K873				
	L904-G905					
				K925-S926		
		K974-G975	K974-G975	K974-G975		
		K992-V993	K992-V993	K992-V993		
		K1040-K1041				
				K1041-V1042		
			R1049-M1050			
		M1076-V1077				
		K1098-M1099	K1098-M1099	K1098-M1099		
		K1136-S1137				
				K1147-N1148		
				K1149-V1150		
				R1310-S1311		
	R1313-A1314	R1313-A1314		R1313-A1314		
		R1422-K1423		R1422-K1423		
				K1473-K1474		
			K1628-Q1629			
			R1648-E1649			
A3-C1 domains				K1673-K1674		
				K1674-E1675		
1649–2172	Y1680-D1681					
			K1693-K1694		+	50
	E1794-D1795				+	20
			K2092-F2093		+	60

[a] Human recombinant FVIII (300 μg) was incubated with affinity-purified anti-FVIII IgG (74 μg) of patient Wal (10) or with unfractionated IgG from the plasma of patients P1 (37.5 μg), CI (37 μg), and I1 (37 μg). Degradation fragments were separated by 10% SDS-PAGE, transferred onto PVDF membranes and sequenced by Edman degradation. Cleavage sites in the C2 domain of FVIII, which are not detected in the assay as fragments lower than 23 kDa, were not subjected to N-terminal sequencing. Amino acids that surround the sites of cleavage are indicated using the single letter code together with the position of the amino acid in the sequence of the processed protein.

[b] Amino acids which are included in the three-dimensional model of FVIII provided on (⟨http://europium.csc.mrc.ac.uk⟩).

[c] Lower limit of the percentage of accessibility of the better of the two amino acids that surround each cleavage site, as determined using SWISS-pdb viewer (⟨http://us.expasy.org/sprot⟩). Amino acids with percentages ≤20 are considered as nonaccessible.

FVIII in Western blots and the purification of IgG to avoid contaminating proteases. If IgG and FVIII could be used at concentrations equal to that used in the Bethesda assay, a FVIII hydrolysis similar to that shown in Fig. 1 would occur in 3.6 min. This hypothesis is strengthened by our earlier observation that affinity-purified anti-FVIII IgG from patients Wal and Bor completely hydrolyzed FVIII in <5 min and 2 h, respectively (10). To formally demonstrate the pathological relevance of FVIII-hydrolyzing IgG, we have modified the Bethesda assay so as to minimize the inhibitory activity of classical FVIII inhibitors: in the presence of HSA, we have used IgG at concentrations yielding >75% residual FVIII activity after 2 h of incubation with FVIII, and we have incubated IgG and FVIII for up to 16 h. In such conditions, the residual FVIII activity decreased in a time-dependent manner when FVIII was incubated with IgG from two patients with FVIII-hydrolyzing Abs, while it remained constant upon incubation of FVIII in the presence of a monoclonal noncatalytic FVIII inhibitor.

Our previous attempt to determine the kinetic parameters of FVIII-hydrolyzing IgG was hampered by the limiting concentration of FVIII (i.e., 1.7 μM), a value far below the calculated average K_m (9.46 ± 5.52 μM) (10). In the present report, PFR-MCA, a synthetic generic substrate for kallikrein and other serine proteases, was used as a surrogate substrate for the precise determination of the catalytic rate constants for the cleavage reaction (14, 25). Several lines of evidence validate PFR-MCA as a proper alternative substrate for studying the kinetics of proteolytic anti-FVIII IgG: 1) anti-FVIII IgG are endowed with a serine-protease-like activity as indicated by the fact that Ab-mediated hydrolysis of FVIII is inhibited by Pefabloc, but not by EDTA, leupeptin, nor pepstatin (10); 2) the hydropathicity of PFR was significantly

Table II. *Cleavage sites for physiological FVIII-hydrolyzing enzymes (32)*

Cleavage Site on FVIII	Serine Protease Involved[a]	Effect	Shared by Patient's IgG
R336	Activated protein C, FIXa, FXa	Inactivation	P1, C1, I1
R372	Thrombin, FXa	Activation	Wal
R562	Activated protein C	Inactivation	
R740	Thrombin, FXa	Activation	P1, C1, I1
R1313	Unidentified endoprotease	Intracellular proteolysis	Wal, P1, I1
R1648	Unidentified endoprotease	Intracellular proteolysis	Wal
R1689	Thrombin, FXa	Activation	
R1719	FIXa	Activation	
R1721	FXa	Activation	

[a] FXa, activated factor X; FIXa, activated factor IX.

correlated to that of 336 of the 2330 tripeptides that constitute the FVIII molecule and to that of 16 of the 45 identified cleavage sites on FVIII; and 3) excess of FVIII neutralized the hydrolysis of PFR-MCA by patients' IgG. PFR-MCA allows the measurement of the capacity of the Abs to hydrolyze an amide bond independently of their affinity for FVIII. PFR-MCA is however inappropriate to judge the specific rates of IgG-mediated hydrolysis of FVIII. Indeed, the rate of FVIII hydrolysis by the Abs is limited by the affinity of the IgG for FVIII. This may explain the lack of correlation between the hydrolytic activity of the Abs toward FVIII and that toward PFR-MCA (Fig. 2).

Based on the derived K_{cat} values, catalytic IgG cleaved 43.2–3758.4 molecules of PFR-MCA over the 24-h period of reaction, indicating that IgG molecules are capable of turnover, a defining feature of catalysts. The average K_m of patients' IgG for PFR-MCA (794.2 ± 300.8 μM) was 80-fold greater than that scored previously when using radiolabeled FVIII as a substrate, i.e., 9.46 ± 5.62 μM (10), indicating that the affinity of anti-FVIII IgG is higher for FVIII than for PFR-MCA. This is reminiscent of previous observations that the active and Ag-binding sites are spatially separated in the V region of hydrolytic IgG (26). The estimated mean catalytic efficiencies ranged from 68.3 to 2682.8/M/min. In normal plasma, FVIII is activated by cleavage by thrombin and FXa, to form a heterotrimer A2/A1/A3-C1-C2 with full procoagulant activity (27). The reported catalytic efficiencies for FVIII cleavage by thrombin and FXa are 5×10^6 and 1×10^6/M/s, respectively (28–31). These values are 10^3- to 10^6-fold higher than the mean catalytic efficiencies that we determined for IgG, thus potentially questioning the pathophysiological relevance of FVIII-hydrolyzing IgG. However, in our calculations we assumed that all anti-FVIII IgG molecules are catalytic, i.e., 17.5 μg of anti-FVIII IgG/mg of IgG (19, 20). Paul et al. (12) have estimated the quantity of catalytic IgG in the plasma of patients with asthma to be 11 ng/mg of IgG. The concentration of catalytic anti-FVIII IgG we have considered may thus be up to 1000-fold overestimated, and the kinetic parameters underestimated to a similar extent. Hence, the cleavage of FVIII by catalytic IgG might follow similar kinetic patterns as that seen with natural enzymes that cleave FVIII.

We identified multiple cleavage sites of FVIII by catalytic Abs: 7, 17, 13, and 25 cleavage sites for IgG of patients Wal, P1, C1, and I1, respectively. Because degradation products that were smaller than 23 kDa could not be sequenced due to technical constraints, scissile bonds located in the C2 domain of FVIII were not detected and the total number of cleavage sites is likely to be underestimated. Multiple cleavage of large proteins by catalytic Abs has already been reported in the case of thyroglobulin and prothrombin (14, 16). Approximately 50% of cleavages occurred at Arg-X bonds that are specific for serine proteases including the conventional coagulation proteases. Thus, some of the identified scissile bonds are also cleavage sites for thrombin, APC, FIXa, FXa, and/or intracellular endoproteases (Table II: R336-M337, R372-S373, R740-S741, R1313-A1314, and R1648-E1649) (32). However, other sites that are typically cleaved by activated protein C (R562-G563), FXa (R1721-A1722), FIXa (R1719-N1720), and thrombin (R1689-S1690) were not detected upon sequencing of the cleavage fragments, further indicating that IgG was not contaminated with conventional proteases. Indeed, the majority of the characterized scissile bonds (40 of 45) are not targets for coagulation proteases. Close to 85% of cleavages occurred after Lys or Arg residues, indicating that most FVIII-hydrolyzing IgG behave as serine proteases and preferentially target peptide bonds that contain basic amino acids.

There was no selectivity of hydrolysis for any particular domain of FVIII; the identified scissile bonds were evenly distributed

Table III. *Catalytic activity of purified IgG and affinity-purified anti-FVIII IgG from the plasma of patients with severe hemophilia A*[a]

Patients	Specific Inhibitory Activity (BU/mg)		Inhibition Index[b]	Specific Hydrolysis Rate of PFR-MCA (pmol/min/nmol)[c]		Activity Index[b]
	IgG	Anti-FVIII IgG		IgG	Anti-FVIII IgG	
B1	28	500–800	18-29	25.01	41.14	2
B2	0.3	57	190	0.13	1.57	12
C1	6	64	11	0.14	2.60	18
N1	7	333	48	0.09	28.62	315
P1	8	500–800	63-100	10.20	44.55	4

[a] IgG were purified from patients' plasma by ammonium-sulfate precipitation and chromatography on Sepharose-coupled protein G. FVIII-specific IgG were further purified by affinity chromatography on a human FVIII matrix. Unfractionated IgG and affinity-purified anti-FVIII IgG were assessed for their specific inhibitory activity towards FVIII in a functional coagulation assay, and for their specific hydrolytic activity towards PFR-MCA.

[b] Defined as the ratio of the values obtained for anti-FVIII IgG and for unfractionated IgG.

[c] Data are representative of two independent experiments.

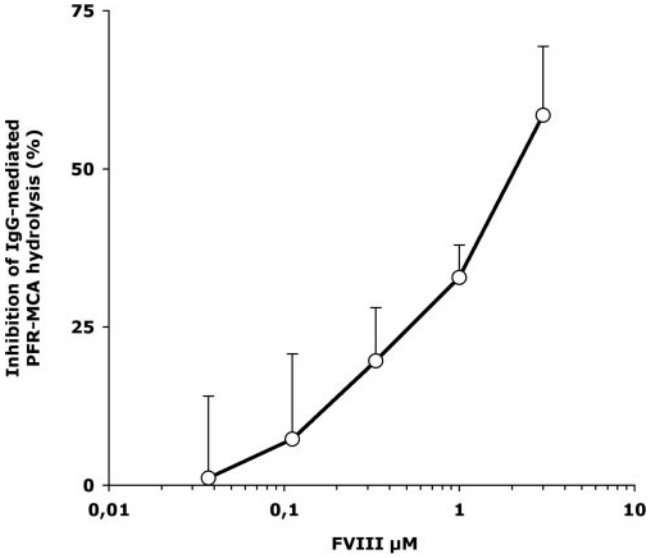

FIGURE 4. Inhibition of IgG-mediated hydrolysis of PFR-MCA in the presence of FVIII. IgG (133 nM) purified from the plasma of patient C1 was allowed to react with increasing concentrations of FVIII (0–3 μM) for 30 min at 37°C. The mixtures were then incubated in the presence of PFR-MCA (60 μM) for 24 h at 37°C. The figure depicts the percentage of PFR-MCA hydrolyzed by IgG in the presence of FVIII as compared with the amount hydrolyzed in the presence of IgG alone. The calculated *K*i was 0.12 ± 0.01 μM (mean from two independent experiments).

within the FVIII molecule. This in contrast with the previously reported clustering of the B cell epitopes on the A2, A3, and C2 domains of the FVIII molecule (Fig. 5; reviewed in Ref. 33). Nine of the 19 cleavage sites included in the three-dimensional model of FVIII (Table I: aa 323, 336, 583, 593, 661, 679, 698, 1693, and 1794) were located within 15 Å of either one of the known B cell epitopes for FVIII inhibitors. It remains to be determined whether multiple cleavages on the FVIII molecule are due to a single polyreactive Ab that hydrolyzes several types of peptide bonds, or to a diverse mixture of catalytic Abs, each with a different binding site and epitope specificity, but all with a structure required for proteolysis. In this respect, multiple cleavage sites for polyclonal IgG of one patient with asthma (34) and for murine and human monoclonal L chains have previously been reported (16, 35). In our study, analysis of the accessibility of the identified scissile bonds reveals

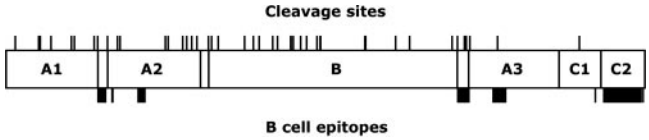

FIGURE 5. Cleavage sites and major B cell epitopes on the FVIII molecule. The distribution of the cleavage sites is based on the results from Table I. The major B cell epitopes are indicated based on the review by Lavigne-Lissalde et al. (33).

that, for each patient, some cleavage sites were located at the surface of the molecule whereas others were not readily accessible. These results suggest that FVIII hydrolysis by patients' IgG occurs in successive steps, i.e., initial cleavages at the level of the exposed sites followed by an opening of the molecule and additional cleavage at the hitherto cryptic sites. Fine temporal analysis of the reaction should allow the precise sorting of events that characterize IgG-mediated hydrolysis of FVIII.

The comparison of the decapeptides that overlap the 45 cleavage sites based on hydropathicity profiles and of the frequency of occurrence of amino acids, did not allow us to identify a consensus linear peptide that could account with certainty for a majority of the identified scissile bonds. However, it may be that some of the cleavage activity would only be neutralized by peptides that mimic conformational rather than linear epitopes, as previously shown for several of the conventional noncatalytic FVIII inhibitors (36, 37). Based on the observations that almost 35% of cleavages occurred after an Arg, that the hydropathicity profile of PFR correlated with 16 of the 45 identified cleavage sites (data not shown) and that MCA-coupled PFR is an appropriate surrogate substrate for FVIII-hydrolyzing Abs (10), it may be speculated that a single or a restricted number of PFR-based tripeptides would be of relevance to block FVIII-specific Ab catalysts. However, their use may require molecular modifications so as to sharpen their broad specificity for different conventional serine proteases and their potential adverse antithrombotic and thrombocytopenic effects (38, 39).

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Table IV. *Heterogeneity of the kinetic parameters of the hydrolysis of PFR-MCA by IgG of severe hemophilia A patients*[a]

Patients	V_{max} (fmol/min)	K_m (μM)	r^2	Mean K_{cat}[b] (min^{-1})	Mean Catalytic Efficiency[c] $(M/min)^{-1}$
B2	14.8 ± 8.4	859 ± 498	0.99	0.06	73.9
B4	6.1 ± 2.3	706 ± 133	0.98	0.03	37.0
C1	13.5 ± 9.1	1418 ± 1248	0.99	0.06	40.8
F1	12.2 ± 2.6	467 ± 125	0.98	0.05	112.0
I1	16.7 ± 3.2	783 ± 308	1.00	0.07	91.4
B1	609.4 ± 178.8	660 ± 247	1.00	2.61	3957.7
P1	384.8 ± 85.7	667 ± 111	1.00	1.65	2472.8

[a] PFR-MCA was incubated at increasing concentrations (0–1.66 mM) with IgG (167 nM) of seven patients with severe hemophilia A, for 24 h at 37°C. Hydrolysis rates were computed as explained in *Materials and Methods.* The data were fitted to the Michaelis-Menten equation and V_{max} *and* K_m were derived. The data represent the mean of two or three independent experiments.

[b] In the calculation of K_{cat}, the quantity of anti-FVIII IgG sites within the pool of IgG was estimated to be equal to 233.3 fM in the assay, based on a ratio of 175 μg of anti-FVIII IgG per 10 mg of IgG (19, 20).

[c] The catalytic efficiency is the ratio of K_{cat} to the corresponding K_m.

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Disclosures

The authors have no financial conflict of interest.

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