THE PREVALENCE OF PROTEOLYTIC ANTIBODIES AGAINST FACTOR VIII IN HEMOPHILIA A

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ABSTRACT

Background Factor VIII inhibitors are IgG alloantibodies that arise during replacement therapy in 25 to 50 percent of patients with severe hemophilia A. The hydrolysis of factor VIII by anti–factor VIII antibodies has been proposed as a mechanism of inactivation of factor VIII.

Methods We purified IgG from patients with severe hemophilia A. The proteolytic activity of the antibodies was assessed by incubating the IgG with biotinylated human factor VIII and analyzing patterns of factor VIII cleavage by sodium dodecyl sulfate–polyacrylamide-gel electrophoresis and immunoblotting. The controls were normal human IgG and IgG purified from plasma of patients with hemophilia who did not have inhibitory antibodies.

Results Significant proteolytic activity was detected in IgG from 13 of 24 inhibitor-positive patients. No hydrolytic activity was detected in control antibodies of IgG from patients without inhibitors. The rate of hydrolysis of factor VIII by purified IgG correlated positively with the factor VIII–neutralizing activity of IgG in plasma ($r^2=0.67$, $P=0.029$). Principal-component analysis of migration profiles of digestion fragments demonstrated the heterogeneity of the catalytic potential of factor VIII inhibitors among patients.

Conclusions Proteolysis is a mechanism by which IgG antibodies against factor VIII can inactivate factor VIII. (N Engl J Med 2002;346:662-7.)

HEMOPHILIA A is an X chromosome–linked recessive hemorrhagic disorder that is characterized by impaired factor VIII production. Antibodies that inhibit factor VIII activity (factor VIII inhibitors) arise in 25 to 50 percent of patients with severe hemophilia A and 5 to 15 percent of patients with mild-to-moderate hemophilia A during treatment with products containing factor VIII.[1,2] Since patients with these alloantibodies become resistant to conventional replacement therapy, the development of inhibitors is a major therapeutic challenge. Factor VIII inhibitors neutralize factor VIII procoagulant activity by sterically preventing the interaction of factor VIII with von Willebrand factor,[3] phospholipids, activated factor IX,[4-6] thrombin, and activated factor X.[7,8]

Catalytic antibodies are immunoglobulins with a capacity to hydrolyze the antigen for which they are specific. The proteolysis of factor VIII by anti–factor VIII alloantibodies has recently been reported as an additional mechanism by which factor VIII inhibitors can neutralize the procoagulant function of factor VIII.[9] In the present study, we investigated the prevalence of catalytic IgG antibodies against factor VIII among patients with severe hemophilia A and inhibitors.

METHODS

Patients

Frozen plasma samples from 24 patients with factor VIII inhibitors and severe hemophilia A (plasma factor VIII levels less than 1.0 percent of normal) were obtained from the Hôpital Cochin (Paris), Hôpital du Kremlin-Bicêtre (Paris), Hôpital de Saint-Etienne (St. Etienne, France), Gasthuisberg Leuven (Leuven, Belgium), and Christian Medical College Hospital (Vellore, India). Most of the patients had received a diagnosis of severe hemophilia within the first two years of life. The mean (±SD) age of the patients was 40.6±18.4 years (range, 10 to 70). Three patients had a low inhibitory titer (less than 5 Bethesda units [BU] per milliliter), and 21 had an inhibitory titer of at least 5 BU per milliliter. Plasma samples were also obtained from four patients with severe hemophilia A and no detectable inhibitor activity in the plasma. Inhibitor-positive patients who had mild hemophilia, or who had severe hemophilia and were undergoing induction of immune tolerance, were not included in the study.

Purification of IgG

IgG was isolated from plasma by ammonium sulfate precipitation followed by chromatography on protein G Sepharose. A therapeutic preparation of pooled normal human immune globulin (Sandoglobulin, ZLB Bioplasma, Bern, Switzerland) was used as a source of normal IgG. To exclude contaminating proteases, size-exclusion chromatography of IgG and immune globulin was performed on a superose-12 column equilibrated with 50 mM TRIS, 8 M urea, and 0.02 percent sodium azide (pH 7.7) at a flow rate of 0.25 ml

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per minute. The IgG-containing fractions were then pooled and dialyzed against 50 mM TRIS, 100 mM glycine, and 0.02 percent sodium azide (pH 7.7) for two days at 4°C. The purity of IgG preparations was assessed by sodium dodecyl sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE) and immunoblotting under nonreducing conditions. IgG was quantified by enzyme-linked immunosorbent assay. In the case of Patient 22, anti–factor VIII IgG antibodies were purified from total IgG by affinity chromatography on a human factor VIII matrix, as previously described.[10]

Evaluation of Factor VIII–Inhibitory Activity

Factor VIII–inhibitory activity was measured in plasma and in purified IgG fractions according to the method of Kasper et al.[11] The detection limit of the Bethesda assay was 0.3 BU.

Hydrolysis of Biotinylated Factor VIII

Commercially available recombinant human factor VIII (Kogenate II, Bayer, Pittsburgh), which contains imidazole, glycine, sucrose, chloride, sodium, calcium, histidine, and polysorbate 80 as stabilizing agents and is free of other proteins, was used in all experiments. After desalting on a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden), factor VIII (3 ml at a concentration of 198 μg per milliliter) in borate buffer (100 mM boric acid [pH 7.0], 150 mM sodium chloride, and 5 mM calcium chloride) was conjugated with biotin (440 μl at a concentration of 25 μg per milliliter) in the dark for two hours at 4°C, as previously described.[12] Biotinylated factor VIII (100 μg per milliliter, 385 mM) was incubated in 40 μl of 50 mM TRIS–hydrochloric acid (pH 7.7), 100 mM glycine, 0.025 percent Tween 20, and 0.02 percent sodium nitrate buffer, alone or in the presence of the IgG to be tested (25 μg per milliliter, 167 nM) for 24 hours at 37°C. Samples were mixed with Laemmli's buffer without mercaptoethanol (1:1 vol/vol). Twenty microliters of each sample was then subjected to 7.5 percent or 10 percent SDS-PAGE. The proteins were transferred onto nitrocellulose (Schleicher and Schuell, Dassel, Germany). After overnight blocking in phosphate-buffered saline, 1 percent bovine serum albumin, and 0.1 percent Tween 20 at 4°C, the membranes were incubated for 30 minutes at room temperature with streptavidin-coupled horseradish peroxidase (Amersham Pharmacia Biotech) diluted 1:3000 in blocking buffer. After being washed in phosphate-buffered saline and 0.1 percent Tween 20, the labeled proteins were revealed with the ECL kit (Amersham Pharmacia Biotech) and BioMax ML films (Kodak, Chalon-sur-Saône, France). The films were scanned with a SnapScan 600 scanner (Agfa, Mortsel, Belgium), and the images were processed with Color It software (MicroFrontier, Des Moines, Iowa). To calculate the rates of factor VIII hydrolysis, the densitometry of the protein bands was computed with National Institutes of Health image 1.62b7 software. Spontaneous hydrolysis occurring on incubation of factor VIII in the presence of buffer alone was considered to represent the background level and was subtracted from each analysis. The significance of the increase in the hydrolytic activity of patient IgG as compared with that of immune globulin (used as a negative control) was assessed by an analysis of variance post hoc test. The reported P values are two-sided.

RESULTS

Inhibitory Activity against Factor VIII

The mean inhibitory activity against factor VIII was 2.7±1.5 BU per milliliter (range, 1 to 4) in the plasma of 3 patients with low titers and 54.6±62.5 BU per milliliter (range, 6.7 to 280) in the plasma of 21 patients with high titers. Inhibitory activity against factor VIII was also measured in purified IgG from the plasma of 21 of the 24 inhibitor-positive patients; in the case of Patients 2, 19, and 20, the quantity of IgG available was insufficient for all the assays. The inhibitory activity of purified IgG ranged between undetectable levels and 10.8 BU per milligram of IgG (Table 1). On average it was 1/10 that found in whole plasma, a result in agreement with an average IgG concentration in plasma of 10 mg per milliliter. There was a significant correlation between inhibitor activity in unfractionated plasma and that in the purified IgG fraction from plasma ($r^2=0.72$, $P<0.001$ by Spearman rank correlation).

Proteolytic Activity against Factor VIII

Figure 1 depicts the electrophoretic pattern of migration of biotinylated factor VIII after incubation

TABLE 1. INHIBITORY ACTIVITY AGAINST FACTOR VIII OF IgG FROM 24 PATIENTS WITH SEVERE HEMOPHILIA A AND RATES OF HYDROLYSIS OF FACTOR VIII.*

PATIENT NO.	INHIBITORY ACTIVITY OF IgG IN PLASMA	INHIBITORY ACTIVITY OF PURIFIED IgG	HYDROLYSIS RATE†
	BU/ml	BU/mg	pmol/min/nmol
1	1	0	0.03±0.05
2	280	ND	1.16±0.19‡
3	10	0.3	0.56±0.14‡
4	12	0.9	0.19±0.01‡
5	110.9	9.5	0.32±0.04‡
6	67.6	6	0.59±0.25‡
7	3	0	0.21±0.00‡
8	9.1	0.4	0.06±0.02
9	99	2.4	0.02±0.01
10	19	0.7	0.02±0.03
11	100	10.5	0.37±0.04‡
12	25.5	1.5	0.16±0.00
13	4	0	0.21±0.06‡
14	6.7	0.7	0.29±0.11‡
15	34	1.2	0.06±0.01
16	40	0.9	0.08±0.05
17	92.3	7.4	0.15±0.04
18	25	2.3	0.14±0.04
19	70	10.8	0.48±0.15‡
20	80	10.1	0.78±0.14‡
21	19	2.7	0.16±0.05
22	16	1.3	0.17±0.02
23	23.5	0.5	0.25±0.01‡
24	8	<0.8	0.33±0.03‡
Immune globulin	0	0	0.04±0.04

*BU denotes Bethesda units, and ND not determined.

†Hydrolysis of factor VIII was quantified by scanning of immunoblots. Spontaneous hydrolysis that occurred on incubation of factor VIII in the presence of buffer alone was considered to represent the background level and was subtracted from each analysis. Plus–minus values are means ±SD of two or three experiments. The mean coefficient of variation was 0.33 (range, 0.01 to 1.41).

‡$P<0.05$ for the comparison with immune globulin.

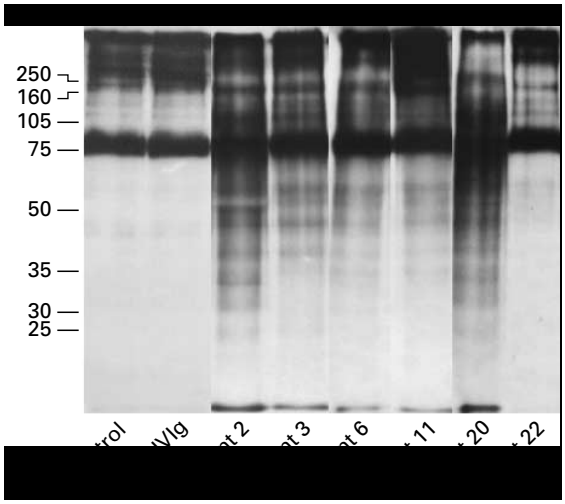


Figure 1. Hydrolysis of Factor VIII by IgG Purified from Plasma of Patients with Severe Hemophilia A.

Biotinylated factor VIII (385 nM) was incubated alone (Control) or in the presence of IgG antibody (167 nM) for 24 hours at 37°C. Immune globulin (IVIg) was used as a source of normal IgG and as a negative control. Samples were separated by 10 percent sodium dodecyl sulfate–polyacrylamide-gel electrophoresis.

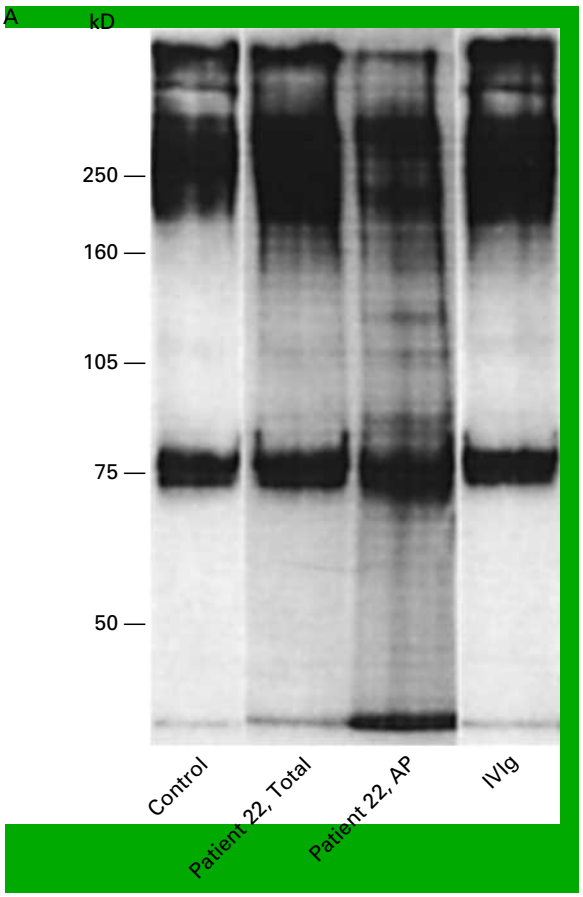
with buffer or antibodies from different sources. Biotinylated factor VIII gave a characteristic pattern of several bands corresponding to molecular masses of 250 to 337 kD and an additional band of 82±2 kD. Incubation of biotinylated factor VIII with IgG purified from the plasma of inhibitor-positive patients caused a detectable cleavage of factor VIII in 14 of the 24 samples. Hydrolysis of factor VIII was most complete in Patients 2, 3, 6, 11, and 20 (Fig. 1). The migration profiles obtained with antibodies from these five patients showed nine cleavage products of 57±1, 50±1, 47±1, 44±2, 38±1, 35±1, 33±1, 30±1, and 27±1 kD. Intermediate cleavage fragments were also observed, with molecular masses greater than 82 kD (Fig. 2A). N-terminal sequencing of the cleaved fragments confirmed that all hydrolysis products were derived from factor VIII (data not shown). The migration profiles of factor VIII that had been incubated with normal human IgG that had no detectable inhibitory activity against factor VIII (Fig. 1), or with IgG from the plasma of patients with hemophilia A who had no inhibitor (data not shown), were similar to that of factor VIII incubated in buffer alone. Four samples of purified IgG that hydrolyzed recombinant factor VIII did not hydrolyze recombinant factor IX or albumin under the same experimental conditions.

The rate of hydrolysis of factor VIII per mole of IgG was calculated after densitometric analysis of the electrophoretic bands (Table 1). Immune globulin had a marginal hydrolytic activity of 0.04±0.04 pmol per minute per nanomole. This value was equal to or, in most cases, lower than the hydrolytic activity scored for IgG of inhibitor-positive patients. Statistical analysis by the analysis-of-variance post hoc test showed that IgG from 13 of 24 patients (Patients 2, 3, 4, 5, 6, 7, 11, 13, 14, 19, 20, 23, and 24) exhibited hydrolytic activity against factor VIII that was significantly greater than that of immune globulin ($P<0.05$ for all comparisons). The average hydrolytic activity of IgG from these 13 patients was 0.44±0.28 pmol per minute per nanomole, whereas that of IgG from patients with hemophilia who did not have inhibitors was 0.04±0.04 pmol per minute per nanomole and did not differ from that of immune globulin (Table 2). Anti–factor VIII IgG antibodies from Patient 22 were isolated from total IgG by affinity chromatography on factor VIII Sepharose. Whereas total IgG from Patient 22 did not have significant hydrolytic activity, the affinity-purified anti–factor VIII IgG did have strong factor VIII hydrolytic activity (Fig. 2A). These data indicate that the factor VIII–neutralizing activity of IgG with inhibitory activity is associated with proteolytic activity against factor VIII. Figure 2B depicts the inhibitory activity against factor VIII in plasma from the 13 patients described above, plotted against the rate of hydrolysis of factor VIII by purified IgG. The two measurements were positively correlated ($r^2=0.67$, $P=0.029$ by Spearman rank correlation), although the correlation is heavily driven by the values of IgG from Patient 2.

We also determined the density of each factor VIII digestion fragment in all 24 samples by scanning electrophoretic gels. The data were subjected to principal-component analysis.[13,14] Principal-component analysis discriminated between the factor VIII digestion profiles of serum samples from two groups of patients: the 13 patients whose IgG hydrolyzed factor VIII at a significantly higher rate than did immune globulin, and the other 11 patients ($P<0.001$ by multivariate analysis of variance) (data not shown). Principal-component analysis also discriminated between the digestion profiles of factor VIII from the 13 patients and that of factor VIII incubated with buffer alone or with immune globulin ($P=0.01$).

DISCUSSION

Several mechanisms have been proposed to account for inactivation of factor VIII in patients with hemophilia A and factor VIII inhibitors.[3-8,15] We recently reported that anti–factor VIII IgG antibodies of two patients with a factor VIII inhibitor could hydrolyze factor VIII.[9] On the basis of the derived catalytic efficiency of these antibodies and the half-life of circulating IgG, we proposed that the hydrolysis of factor



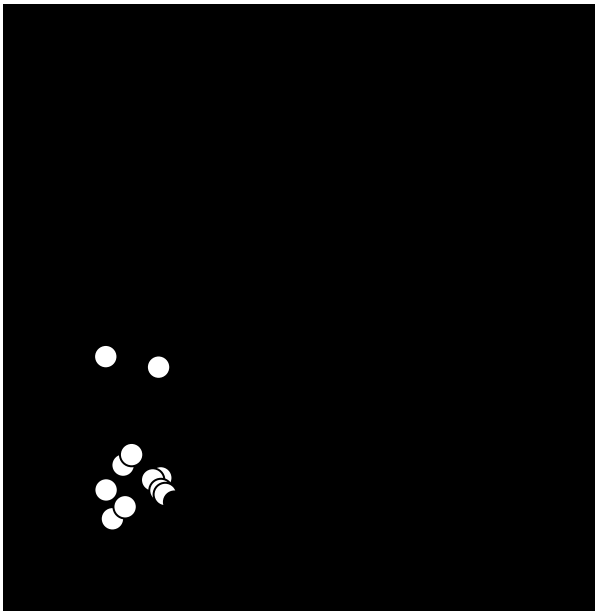


Figure 2. Hydrolysis of Factor VIII by IgG of Patients with Severe Hemophilia A.

Panel A shows the results after biotinylated factor VIII (385 nM) was incubated alone (Control) or in the presence of immune globulin (IVIg), IgG purified from plasma from Patient 22 (Total), or affinity-purified anti–factor VIII IgG antibodies from Patient 22 (AP), at 167 nM for 24 hours at 37°C. Samples were separated by 7.5 percent sodium dodecyl sulfate–polyacrylamide-gel electrophoresis. Panel B shows the correlation between the inhibitory activity of purified IgG against factor VIII and the rate of hydrolysis of factor VIII by purified IgG. Data were plotted for all 24 patients: solid circles represent the 13 patients whose IgG exhibited marked proteolytic activity against factor VIII (only 12 solid circles are visible because 2 are overlapping), and open circles represent the 11 patients with no detectable factor VIII–hydrolyzing IgG. The results from the linear regression analysis of the data are indicated by the dashed line for all 24 patients and by the solid line for the 13 patients with factor VIII–hydrolyzing IgG. The significance of the correlations between factor VIII–inhibitory activity and the rate of factor VIII hydrolysis was assessed by nonparametric Spearman rank correlation. BU denotes Bethesda units.

VIII may account, at least in part, for the rapid elimination of factor VIII from the plasma after the infusion of factor VIII into patients with factor VIII inhibitors.[9] We found that 13 of 24 unselected patients with factor VIII inhibitors also had catalytic antibodies against factor VIII in their plasma. The correlation between the factor VIII–hydrolyzing activity of IgG and the factor VIII–neutralizing activity in plasma was statistically significant but not definitively established.

The prevalence of proteolytic antibodies found in our study may be an underestimate, since an isolated population of anti–factor VIII antibodies from the plasma of a patient with no detectable hydrolytic activity in the total IgG fraction (Patient 22) had hydrolytic activity against factor VIII. We have previously documented that the proteolytic activity of anti–factor VIII IgG is mediated by $F(ab')_2$ fragments, that the purified IgG is free from contaminating proteases, and that the hydrolytic activity of the antibodies is not inhibited by a broad range of generic protease inhibitors.[9,16,17] In the present study, the purification of IgG on protein G Sepharose, followed immediately by size-exclusion chromatography in 8 M urea, made it unlikely that the hydrolysis of factor VIII was caused by contaminating proteases.

Hydrolytic antibodies against vasoactive intestinal peptide, DNA, thyroglobulin, and prothrombin have been described in asthma,[18,19] systemic lupus erythematosus,[20,21] autoimmune thyroiditis,[16] and multiple myeloma.[17] The clinical relevance of such antibodies is, however, unclear. In hemophilia, by contrast, the association between the hydrolytic activity of antibodies against factor VIII and clinical manifestations (resistance to replacement therapy) is plausible.

TABLE 2. HYDROLYTIC ACTIVITY AGAINST FACTOR VIII OF IgG FROM FOUR PATIENTS WITH SEVERE HEMOPHILIA BUT NO INHIBITOR.

PATIENT NO.	HYDROLYSIS RATE*	P VALUE†
	pmol/min/nmol	
25	0.01±0.00	0.68
26	0.01±0.00	0.73
27	0.05±0.03	0.88
28	0.09±0.06	0.39
Immune globulin	0.04±0.04	—

*Hydrolysis of factor VIII was quantified by scanning of immunoblots. Spontaneous hydrolysis occurring on incubation of factor VIII in the presence of buffer alone was considered to represent the background level and was subtracted from each analysis. Plus–minus values are means ±SD of two or three experiments. The mean coefficient of variation was 0.60 (range, 0.03 to 1.44).

†P values are for the comparison with immune globulin.

Patients with hemophilia A constitute a heterogeneous group. In patients with mild-to-moderate or severe hemophilia A, factor VIII inhibitors can develop during treatment with human factor VIII. The probability of developing factor VIII inhibitors is greater in patients with large deletions in the factor VIII gene.[22] We restricted our analysis to patients with severe hemophilia A and factor VIII–neutralizing activity in plasma. The relation between the hydrolytic activity of IgG and factor VIII–neutralizing activity was not consistent. Antibodies from some patients caused hydrolysis of factor VIII at low rates, but the plasma had strong inhibitory activity (Patients 9 and 16); in other cases, the IgG caused hydrolysis of factor VIII at high rates, but the plasma had weak inhibitory activity (Patients 3 and 14); in three samples, there were high rates of both hydrolysis and inhibitory activity (Patients 2, 11, and 20). This inconsistency may be a reflection of multiple mechanisms that participate simultaneously in inactivating factor VIII. Principal-component analysis of the results of factor VIII digestion, which takes into consideration the amount of factor VIII hydrolyzed per unit of time and the size of the proteolytic products, distinguished two groups of patients among the 13 patients, further emphasizing the heterogeneity of inhibitors in severe hemophilia A.

Sites on factor VIII that may be common targets of proteolytic anti–factor VIII antibodies are currently under investigation. Such studies may provide a rationale for the design of novel approaches to the treatment of patients with factor VIII inhibitors with hydrolytic activity.

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