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Natural antibodies to factor VIII (anti-hemophilic factor) in healthy individuals

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ABSTRACT **Spontaneous inhibitors of factor VIII (FVIII) are pathogenic IgG autoantibodies of restricted isotypic heterogeneity found in the plasma of patients presenting with bleeding episodes and low levels of FVIII. We now report the presence of a natural FVIII-neutralizing activity in 85 of 500 plasma samples (17%) from healthy donors. FVIII-inhibitory activity was present in $F(ab')_2$ fragments of purified IgG and was dose-dependent. The titer of anti-FVIII antibodies in normal plasma ranged between 0.4 (threshold of detection) and 2.0 Bethesda units. Anti-FVIII IgG was also detected in normal plasma by using an ELISA. Anti-FVIII antibodies from healthy individuals did not exhibit restricted isotypic heterogeneity. Mean levels of FVIII activity did not differ significantly between individuals with and without detectable anti-FVIII antibodies in plasma. Natural anti-FVIII IgG inhibited FVIII activity in pools of normal plasma and in plasma of certain donors in the pool but did not inhibit FVIII activity in autologous plasma. These observations demonstrate that polyclonal IgG antibodies against procoagulant FVIII are present in healthy individuals. The antibodies are natural IgG autoantibodies and/or antibodies directed against epitopes associated with a so far unidentified allotypic polymorphism of the human FVIII molecule.**

The presence of autoantibodies to factor VIII (FVIII) has so far exclusively been investigated in the plasma of patients with spontaneously occurring severe bleeding episodes in whom circulating autoantibodies are associated with low levels of FVIII. The inhibitors are IgG autoantibodies of restricted isotypic heterogeneity (1) that bind to selective sites on the FVIII molecule, resulting in inhibition of FVIII procoagulant activity (2–4).

Natural IgM and IgG autoantibodies reacting with a wide array of serum proteins and hormones, and nuclear and cellular antigens are found in normal human serum (5). In the present study, we found that the plasma of 17% of 500 healthy blood donors contained FVIII-neutralizing activity. Anti-FVIII activity was present in $F(ab')_2$ fragments from the IgG fraction of the plasma samples with inhibitor activity. FVIII-neutralizing activity of anti-FVIII IgG from a given donor differed with the individual source of FVIII used in the neutralization assay. These observations demonstrate the presence of natural autoreactive and/or alloreactive IgG antibodies to FVIII in healthy individuals.

METHODS

Blood Donors and Sample Collection. Whole blood was obtained from 500 regular volunteer blood donors. The donors included 250 females and 250 males. Male and female donors were equally distributed among five groups from 18 to 26, 27 to 35, 36 to 44, 45 to 53, and 54 to 65 years of age. The donor population was Caucasoid and showed the expected frequency of ABO phenotypes in the European Caucasian population.

Blood was collected in 0.13 M sodium citrate, 1:9 (vol/vol), and immediately centrifuged at 2500 × *g* for 15 min at room temperature. Plasma was divided and stored at −80°C for no longer than 4 weeks.

Pooled plasma and IgG from the 420 healthy donors lacking detectable anti-FVIII activity in plasma were used as negative controls.

FVIII Assays. FVIII activity was measured in a one-stage assay by a manual activated partial thromboplastin reagent (APTT) method using human plasma depleted of FVIII (containing <1% FVIII and normal levels of factor V; coagulation time in APTT, >200 sec) (General Diagnostics, Morris Plains, NJ) as substrate and human brain partial thromboplastin and Kaolin (5 mg/ml) as activators. The clotting time of four serial dilutions (1:20 to 1:160) of a reference plasma pool was compared to the clotting time of the same dilutions of each plasma sample. Each plasma sample was tested in duplicate. Dilutions were carried out in barbital-buffered saline (BBS). The reference plasma pool was prepared with plasma from 20 healthy individuals and calibrated with a standard of FVIII (obtained from T. W. Barrowcliffe, National Institute for Biological Standards, London, U.K.). All assays were performed by the same experienced investigator. Interassay variations ranged between 1 and 2.5% as calculated from five assays performed on the same plasma on 10 occasions. von Willebrand activity was measured using a standard ristocetin cofactor assay.

Quantitation of Anti-FVIII Activity. FVIII-neutralizing activity was measured using the method of Kasper *et al.* (6) and expressed in Bethesda units (BU). To remove FVIII activity from test samples, plasma was heated for 1 h at 56°C, left for 15 min at room temperature, and centrifuged at 2500 × *g* for 15 min prior to determination of anti-FVIII activity (7). Based on the confidence limits of measurements of FVIII activity in the laboratory, a decrease of ≥25% in FVIII activity in the reference pool incubated with the test sample as compared with residual FVIII activity in the pool incubated with BBS alone was considered as significant. Seventy-five percent residual FVIII activity (mean + 2.5 SEM) in the test sample corresponded to 0.4 BU and defined the threshold of positivity for the detection of anti-FVIII activity. When residual FVIII activity was <25%, the test sample was diluted until a value of residual FVIII close to 50% was obtained in the anti-FVIII assay, as recommended by Kasper and Ewing (8).

Preparation of IgG and $F(ab')_2$ Fragments. The IgG fraction was obtained from plasma by anion-exchange chromatography on DEAE-Trisacryl (IBF, Villeneuve la Garenne, France). $F(ab')_2$ fragments were prepared from IgG by pepsin

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Abbreviations: BU, Bethesda unit(s); FVIII, factor VIII (anti-hemophilic factor); HSA, human serum albumin.

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digestion, 2% (wt/wt) (Sigma), in 0.2 M sodium acetate (pH 4.1) for 18 h at 37°C and by chromatography on protein A-Sepharose (Pharmacia).

ELISA for Anti-FVIII Antibodies. Highly purified FVIII (Hemofil M) obtained from Hyland Baxter (Glendale, CA) was used for the detection of anti-FVIII antibodies by ELISA. FVIII diluted to 20 units/ml in 0.1 M carbonate/0.1 M bicarbonate (pH 9.5) was used to coat ELISA plates (Central European Biotechnology, Angers, France) for 150 min at room temperature. Plates were saturated with 1.0% gelatin in phosphate-buffered saline (PBS) for 90 min at room temperature and washed with PBS. Heat-treated plasma to be tested was diluted with PBS containing 1.0% human serum albumin (HSA) and added to the wells. The plates were then incubated for 2 h at room temperature before extensive washing and addition of peroxidase-labeled goat anti-human IgG antibodies (Cappel Laboratories) or peroxidase-labeled mouse monoclonal antibodies against human IgG subclasses 1, 2, 3, and 4 (The Binding Site, Birmingham, U.K.).

By using the coating conditions described above for Hemofil M, FVIII activity was shown to be present in the wells by using a chromogenic substrate assay (Stachrom VIII:C, Stago Diagnostic, Asnières, France). Incubation of the wells with 1.0 unit of Hemofil M resulted in effective deposition of 0.264 unit of FVIII activity per well. The activity was inhibited if wells had further been incubated with IgG (10.7 mg/ml) from the plasma of a healthy donor with anti-FVIII antibodies. No inhibition occurred if wells were incubated with IgG (10 mg/ml) from a donor without detectable anti-FVIII activity in plasma.

Western Blot Analysis. For Western blot analysis, Hemofil M was chromatographed on an Affi-Gel Blue column (Bio-Rad) to decrease the albumin content of the preparation and electrophoresed (SDS/PAGE) on 7.5% gels. Electrophoresed material appeared to have several high molecular mass bands between 210 and 110 kDa and three bands of 94, 80, and 72 kDa. Electrophoresed proteins were transferred onto a nitrocellulose sheet and incubated with purified IgG from individuals with and without anti-FVIII activity in plasma. Bound antibodies were identified by incubating with peroxidase-labeled goat anti-human IgG Fc antibodies (The Binding Site, Birmingham, U.K.) overnight at 4°C.

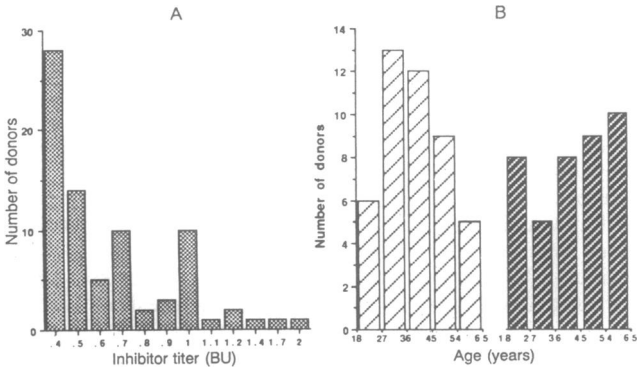

FIG. 1. (*A*) Histogram distribution of the number of donors with anti-FVIII activity in plasma as a function of the inhibitor titer. Eighty-five of 500 plasma samples that were tested contained detectable anti-FVIII activity. (*B*) Histogram distribution of the number of donors with anti-FVIII activity in plasma as a function of age and sex. Open hatched bars, female blood donors (18% of 250 donors had detectable anti-FVIII activity); solid hatched bars, male donors (16% of 250 donors had detectable anti-FVIII activity). For female and male donors, bars represent the number of positive plasma samples among donors aged 18–27 years, 27–36 years, 36–45 years, 45–54 years, and 54–65 years. Fifty donors in each age group were screened for anti-FVIII activity in plasma.

Table 1. Anti-FVIII activity in $F(ab')_2$ fragments from IgG of three normal donors with FVIII inhibitor

Donor	Anti-FVIII activity in plasma, BU/ml	Anti-FVIII $F(ab')_2$ activity, BU/ml	Specific anti-FVIII $F(ab')_2$ activity, BU/mol ($\times 10^{-6}$)
1	0.4	0.3	10.0
2	1.0	2.0	35.0
3	0.4	0.9	27.2
4	—	—	—
5	—	—	—

RESULTS

FVIII-Neutralizing Activity in Plasma from Healthy Donors. Eighty-five of the 500 plasma samples obtained from healthy blood donors (17%) contained detectable FVIII-neutralizing activity of ≥0.4 BU. Anti-FVIII activity was between 0.4 and 2.0 BU. The same sample with inhibitor activity was reproducibly positive (up to 3 months of storage at 20°C). The frequency distribution of plasma samples containing FVIII-neutralizing activity as a function of anti-FVIII activity is shown in Fig. 1. Fig. 1 also shows the frequency distribution of the number of donors with anti-FVIII activity in plasma, according to age and sex. In females, the distribution of donors with inhibitor as a function of age was Gaussian, peaking between 27 and 44 years of age. In males, the number of donors with inhibitor was equally distributed among donors of various age, except for a lower frequency of donors with inhibitor between 27 and 34 years of age.

O group blood donors, which represent 43% of the normal Western European population, represented 67% of 85 individuals with neutralizing activity to FVIII from our study.

Characterization of FVIII Inhibitors in Normal Plasma as IgG Antibodies. Anti-FVIII activity was present in purified IgG from donors with inhibitor, whereas it was absent in IgG from normal individuals with no detectable anti-FVIII activity in plasma (Table 1). Inhibition of FVIII activity by IgG that had been purified from the plasma of a donor with inhibitor activity was dose-dependent (Fig. 2). Anti-FVIII

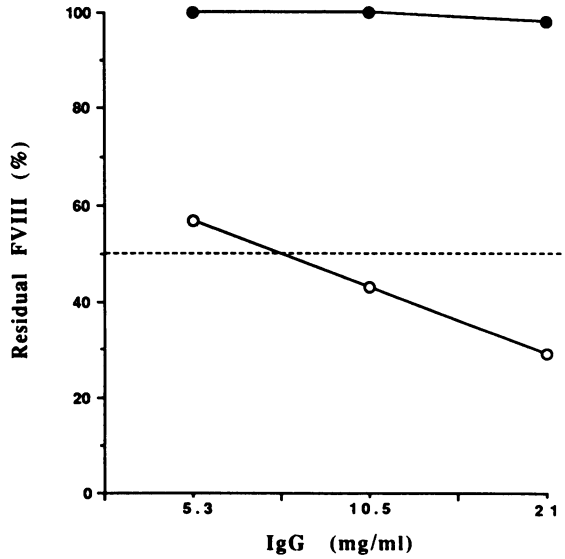

FIG. 2. Dose dependency of anti-FVIII activity in the IgG fraction purified from the plasma of a donor with detectable inhibitor activity. The horizontal line represents 50% of residual activity in the FVIII assay, which defines 1 BU of FVIII activity. ○, IgG from a donor with anti-FVIII activity in plasma; ●, pooled IgG from 420 donors selected for the absence of detectable anti-FVIII activity in plasma.

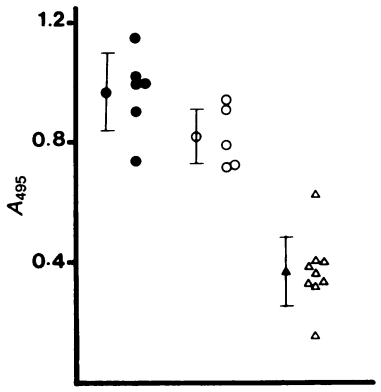

FIG. 3. Detection of anti-FVIII antibodies in the plasma of healthy individuals by ELISA. ●, Anti-FVIII IgG antibodies in plasma from healthy individuals with inhibitor titers of ≥0.7 BU; ○, anti-FVIII IgG in plasma of individuals with inhibitor titers of 0.4 BU; △, healthy individuals with no detectable anti-FVIII activity in plasma (i.e., <0.4 BU); error bars, mean ± SD of values obtained in each group.

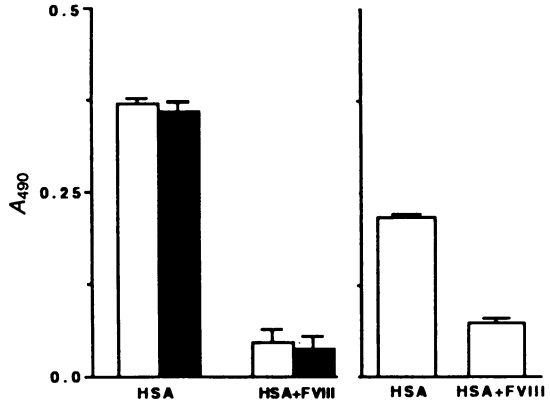

FIG. 5. Inhibition by FVIII of the binding of anti-FVIII antibodies to FVIII. (*Left*) Plasma from a patient with anti-FVIII autoimmune disease (300 BU of inhibitor activity) was diluted 1:250 in 1.0% HSA or in 1.0% HSA plus FVIII (60 units/ml) and incubated overnight at 4°C. The specific binding of IgG1 (open bars) and IgG4 (solid bars) to FVIII was then assessed by ELISA. (*Right*) Same experiment performed with plasma from a healthy donor with IgG1 anti-FVIII activity (0.8 BU) diluted 1:2 in 1.0% HSA or in 1.0% HSA plus FVIII (60 units/ml).

activity was also found in $F(ab')_2$ fragments of IgG. Thus, the inhibitory activity to FVIII that may be found in plasma from healthy individuals represents a specific antibody activity that belongs at least to antibodies of the IgG class.

The presence of IgG against FVIII in plasma of healthy donors was also shown by an ELISA with highly purified FVIII. The ELISA discriminated between normal plasmas with no detectable anti-FVIII activity and plasmas containing a FVIII-neutralizing activity of ≥0.4 BU (Fig. 3). For positive samples, no correlation was found between the titer of anti-FVIII antibodies in plasma measured by a functional assay and the amount of anti-FVIII antibodies determined by ELISA (Fig. 3). Anti-FVIII autoantibodies from healthy individuals detected by ELISA belonged to the four sub-

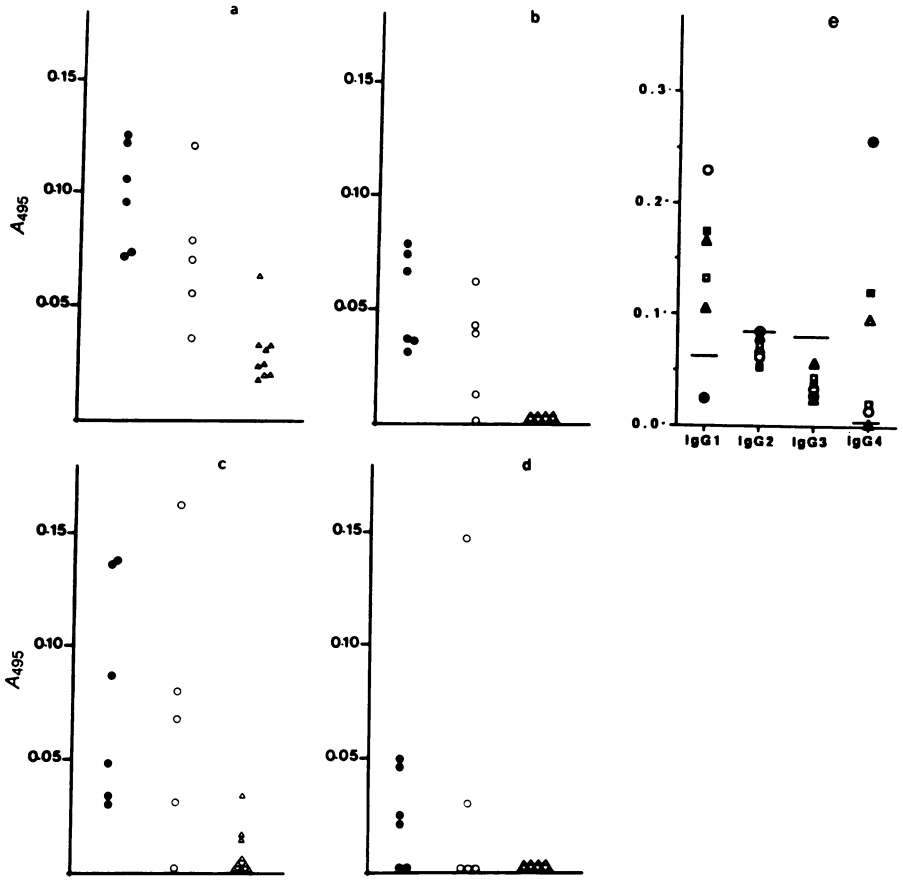

FIG. 4. Isotypic distribution of anti-FVIII IgG antibodies. (*a–d*) Anti-FVIII IgG in plasma from healthy individuals. Symbols are as in Fig. 3. (*a*) IgG1. (*b*) IgG2. (*c*) IgG3. (*d*) IgG4. (*e*) Anti-FVIII IgG in the plasma from six patients with anti-FVIII autoimmune disease. The amount of IgG in each ELISA well was adjusted so that the wells would contain the same amount of anti-FVIII activity. Bars indicate the threshold of negativity of the ELISA defined with a pool of IgG from donors lacking anti-FVIII activity in plasma. Each symbol represents a patient tested for the isotypic profile.

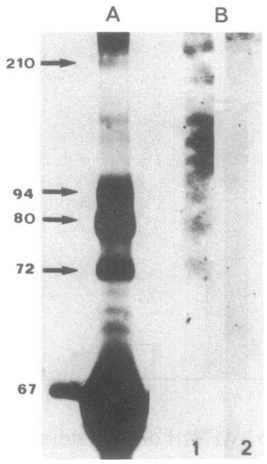

FIG. 6. Western blot analysis. (*A*) SDS/PAGE of Affi-Gel Blue-purified Hemofil M; residual albumin is seen at 67 kDa. (*B*) Blot of pooled purified IgG from 20 healthy donors with anti-FVIII activity in plasma (25 mg/ml; lane 1) and pooled IgG from 420 donors lacking anti-FVIII activity in plasma (25 mg/ml; lane 2). Molecular masses in kDa are shown.

classes of IgG whereas antibodies of patients with anti-FVIII autoimmune disease belonged to the IgG1 and IgG4 classes (Fig. 4). The specificity of the ELISA for FVIII was demonstrated by the following experiment: treated plasma was diluted either in PBS containing 1.0% HSA or in PBS containing 1.0% HSA and FVIII at 60 units/ml. After incubation overnight at 4°C, an ELISA was performed. Incubation of treated plasma with FVIII inhibited the binding of IgG to FVIII-coated plates (Fig. 5).

Western blot analysis demonstrated that IgG from healthy donors with anti-FVIII activity in plasma recognized predominantly three bands of highly purified FVIII with molecular masses between 210 and 110 kDa and, to a lesser extent, the bands at 94, 80, and 72 kDa (Fig. 6); the IgG did not recognize albumin.

FVIII Levels in Normal Individuals with Anti-FVIII Antibodies. The mean level of FVIII activity in plasma did not differ significantly between individuals with and without detectable anti-FVIII activity in plasma (Fig. 7). Plasma

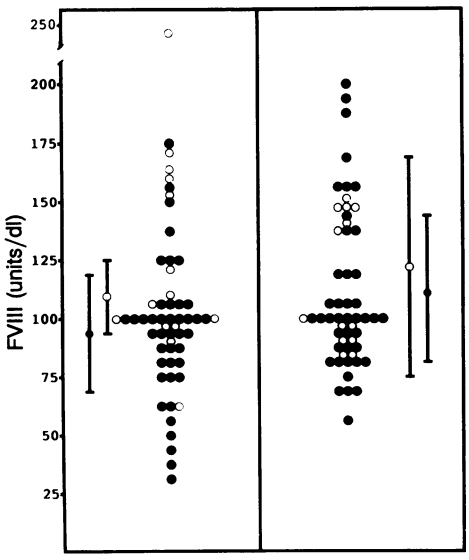

FIG. 7. Levels of FVIII in plasma samples from healthy females (*Left*) and males (*Right*) with (solid circles) and without (open circles) detectable anti-FVIII activity in plasma. Bars indicate the mean ± SEM.

Table 2. Anti-FVIII activity of IgG from healthy individuals toward FVIII in plasma from various donors

	Anti-FVIII activity, BU/mol of IgG ($\times 10^{-6}$)				
Donor	A	B	C	D	E
1	—	—	—	—	—
2	—	—	—	—	—
3	—	—	—	—	—
4	2.6	2.6	3.3	6.8	12.9
5	—	—	—	—	—
6	—	2.6	—	—	9.7
7	3.5	5.2	5.5	9.1	19.3
8	—	—	—	—	—
9	—	2.6	—	—	12.9
10	—	—	—	—	—
11	—	—	—	—	—
12	—	—	—	—	—
13	—	—	—	—	—
14	—	—	—	—	—
15	—	—	—	—	—
16	—	2.6	3.3	—	12.9
17	—	—	—	—	—

Purified IgG from 5 healthy donors (A–E) with anti-FVIII antibodies was incubated with a pool of plasma from 17 healthy individuals without detectable inhibitor to FVIII and with each of the 17 plasma samples constitutive of the pool. Residual FVIII activity was then assessed and expressed as anti-FVIII activity of the IgG. Concentration of IgG from donors A–E that was incubated with a fixed volume of the plasma pool and of plasmas from individual donors 1 to 17 was as follows: A, 141 nmol/ml; B, 115 nmol/ml; C, 91 nmol/ml; D, 44 nmol/ml; E, 31 nmol/ml. —, Below the threshold of detection (i.e., <0.4 BU).

levels of FVIII ranged between 60 and 250 units/dl (mean ± SEM = 107 ± 19 units/dl) in 14 males with anti-FVIII antibodies and between 85 and 130 units/dl (125 ± 47 units/dl) in 14 males without inhibitor (not significant). Levels of FVIII ranged from 30 to 175 units/dl (95 ± 28 units/dl) in females with anti-FVIII antibodies and from 60 to 200 units/dl (109 ± 34 units/dl) in females without inhibitor (not significant). Four healthy females with FVIII inhibitor had FVIII levels below 50 units/dl. Levels of von Willebrand activity in plasma of two of these four female donors in whom it could be measured were 66% and 116%. Purified IgG from five healthy individuals with anti-FVIII antibodies was tested for its ability to neutralize FVIII activity in the plasma of 17 normal donors with no detectable anti-FVIII antibodies after a 2-h incubation at 37°C. The amount of plasma was adjusted in each assay so that the same amount of FVIII activity was added to the IgG that was tested. IgG from the five healthy individuals with anti-FVIII antibodies neutralized FVIII activity in the plasma from 2 of the 17 donors. It did not neutralize FVIII activity in the plasma from 12 of the 17 donors. IgG from each of the five healthy individuals reacted differently with FVIII in plasma from the three remaining

Table 3. Anti-FVIII activity in heated plasma and purified IgG from healthy donors measured using a reference pool of normal plasma or autologous plasma

		Anti-FVIII activity, BU	
Source of antibodies	Donor	Plasma pool	Autologous plasma
Plasma	1	1.70	0
	2	1.00	0
	3	1.70	0
	4	2.20	0
	5	1.20	0
	6	0.45	0
IgG	1	1.20	0

IgG was at 13.6 mg/ml.

donors (Table 2). In similar experiments, IgG from individuals with anti-FVIII activity did not neutralize FVIII activity in autologous plasma (Table 3).

DISCUSSION

The present study indicates that IgG antibodies to FVIII may be present in healthy individuals with normal levels of FVIII in plasma. We have screened normal plasmas for the presence of FVIII-neutralizing activity by using heat-treated plasma to inactivate intrinsic FVIII activity. The source of FVIII in the FVIII-neutralization assay was a plasma pool from 20 healthy donors, as recommended (8). Seventeen percent of 500 regular blood donors were found to exhibit FVIII inhibitory activity in plasma. Purified IgG from plasma with FVIII-neutralizing activity showed dose-dependent inhibitory activity against FVIII. Inhibition of FVIII activity was also found in $F(ab')_2$ fragments from IgG, indicating that FVIII-neutralizing activity is dependent on the antigen-combining site of IgG antibodies.

A similar proportion among male and female donors displayed anti-FVIII antibodies in plasma. The frequency distribution of plasmas containing FVIII inhibitor as a function of age differed between male and female donors with a tendency to peak among women in age groups with higher frequency of pregnancy. There was a tendency toward an increased frequency of O group individuals among donors with natural anti-FVIII antibodies. This observation may be of interest with regard to previous findings of low levels of FVIII in O group blood donors (9, 10).

IgG antibodies to FVIII in donor plasma could also be detected using an ELISA. The ELISA clearly discriminated between positive (>0.4 BU) and negative (<0.4 BU) samples for anti-FVIII activity. For positive samples, the absorbance values determined by ELISA did not correlate with anti-FVIII activity measured in a functional assay. The lack of correlation that was observed between the titers of FVIII-neutralizing activity and the amounts of anti-FVIII IgG determined by ELISA could be due to anti-FVIII antibodies recognizing epitopes located outside the functional sites on the FVIII molecule and/or to the presence of anti-FVIII antibodies of non-IgG isotype. The binding of anti-FVIII antibodies to FVIII was further documented by Western blot analysis.

Natural antibodies of the IgM, IgA, and/or the IgG isotype are found in the plasma of healthy individuals directed against a variety of self-related antigens including idiotypes of anti-FVIII (11, 12) and other autoantibodies (13, 14). The biological significance of natural autoantibodies is not yet understood although there is evidence that the antibodies may serve immunoregulatory functions, have a role in protein and cell catabolism, and contribute to host defense (5). Natural preimmune autoantibodies in the serum of normal individuals are not associated with clinical signs of disease. Whether autoantibodies that are found in the plasma from patients with autoimmune diseases represent an expansion of naturally occurring autoreactive clones or somatically mutated antigen-driven clones is a matter of debate (15). The observation of normal FVIII levels in healthy individuals with anti-FVIII antibodies confirms the lack of correlation between the concentration and affinity of autoantibodies and clinical signs of disease. Mean levels of FVIII activity in the plasma of healthy individuals with anti-FVIII antibodies did not differ from the levels measured in individuals with no detectable FVIII-neutralizing activity, suggesting that anti-FVIII antibodies from healthy individuals express no FVIII-neutralizing capacity toward their own FVIII but express an inhibitory activity toward FVIII in the plasma pool that serves to screen for the presence of antibodies. Autologous FVIII was not inhibited by IgG from healthy individuals with FVIII inhibitory activity in plasma whereas the IgG neutralized some but not all allogeneic sources of FVIII. Some of the anti-FVIII IgG antibodies found in normal plasma would thus represent antibodies against allogeneic rather than autologous antigenic determinants. The presence of anti-FVIII antibodies may thus provide an explanation for the frequent finding of a low yield of FVIII activity in preparations of a therapeutic cryoprecipitate that contain up to 30% of IgG on a weight basis. Low levels of FVIII that were observed in four healthy females with anti-FVIII antibodies could be due to the following factors: (*i*) heterozygous von Willebrand disease, as indicated by a ristocetin cofactor activity of 60 units/dl in one of these women; (*ii*) carriers for hemophilia A; or (*iii*) alternatively, the autoantibodies found in normal individuals could behave in rare instances in a similar fashion to the autoantibodies that occur in individuals with overt anti-FVIII autoimmune disease.

Thus, anti-FVIII antibodies may be detected in the plasma from almost 20% of healthy individuals. The antibodies may be natural IgG autoantibodies and/or antibodies directed against epitopes associated with a so far unidentified allotypic polymorphism of human FVIII.

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