Origin of Anti-idiotypic Activity Against Anti-factor VIII Autoantibodies in Pools of Normal Human Immunoglobulin G (IVIg)

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Therapeutic preparations of polyclonal IgG obtained from plasma pools of a large number of normal donors (IVIg) express anti-idiotypic activity against a wide spectrum of natural and disease-associated autoantibodies. The present study investigated the origin of anti-idiotypic activity against autoantibodies to factor VIII. The neutralizing activity of pools of IgG against patients' anti-factor VIII autoantibodies was not influenced by the presence of individuals with natural anti-factor VIII antibodies among donors contributing to the pool. A higher frequency of neutralizing antibodies against anti-factor VIII autoantibodies was found in aged donors as compared with young adults and in pools of IgG from multiparous women as compared with IgG from randomly selected groups of donors. Pooling IgG from several donors synergistically enhanced the inhibitory activity of the pools. Thus, a neutralizing activity against anti-factor VIII autoantibodies was detected in pools of IgG of as few as two to four donors of whom individually tested IgG did not exhibit inhibitory activity against anti-factor VIII autoantibodies. These observations suggest that aged donors and multiparous women may be privileged sources for the anti-idiotypic activity of IVIg against autoantibodies and emphasize that the expression of anti-idiotypic activity in IVIg results from a synergistic participation of anti-idiotypes from each donor contributing to the pool.

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PATIENTS AND METHODS

Source of anti-factor VIII autoantibodies. Plasma samples were obtained from nine patients with spontaneous inhibitors to factor VIII. Titers of anti-factor VIII antibodies in plasma ranged between 9 and 940 Bethesda Units (BU).

Sources of normal IgG. Serum was obtained from 13 healthy men between 20 and 30 years of age, 14 healthy men between 50 and 65 years of age, 7 nulliparous and 8 multiparous healthy women (aged between 20 and 38 years). None of these donors exhibited natural anti-factor VIII activity in plasma. Plasma pools were prepared from plasma of 500 healthy multiparous women, 500 healthy nulliparous women, and 420 healthy male blood donors who had been selected on the basis of the lack of detectable natural anti-factor VIII activity in plasma.

IgG was purified from plasma pools and from serum by anion exchange chromatography on DEAE Trisacryl (IBF, Villeneuve la Garenne, France) using Tris HCl buffer pH 8.8. When required, F(ab')2 fragments were prepared from IgG by pepsin digestion (2% wt/wt) (Sigma Chemical Co, St Louis, MO) in acetate buffer pH 4.1 for 18 hours at 37°C and chromatography on protein A Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). F(ab')2 fragments were free of detectable IgG as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

IVIg were Sandoglobulin (Sandoz SA, Basel, Switzerland); Endobulin (Immuno AG, Vienna, Austria); Partobulin (Immuno AG); and Veinoglobulin (Institut Mérier, Lyon, France).

Concentrations of purified IgG and F(ab')2 were determined spectrophotometrically at 280 nm using an extinction coefficient of 1.4. The concentration of IgG in serum and plasma was determined by nephelometry.

Assay for anti-factor VIII activity. Anti-factor VIII activity was measured by assessing the ability of autoantibodies to neutralize factor VIII procoagulant activity in a normal reference plasma pool as described by Kasper et al.

Factor-VIII activity was determined in a one-stage activated partial thromboplastin time (APTT) assay using human plasma depleted in factor VIII (containing less than 1% factor VIII and normal levels of factor V; coagulation time in APTT > 200 seconds) (General Diagnostics, Durham, NC) 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 with the clotting time of the same dilutions of each test sample. Each test sample was tested in duplicate. Dilutions were performed in barbitral-buffered saline (BBS). The reference plasma pool was prepared with plasma from 20 healthy individuals and calibrated with a standard of factor VIII (obtained from Dr T.W. Barrowcliffe, National Institute for Bio-
logical Standards, London, UK). All assays were performed by the same investigator. Inter-assay variations ranged between 1% and 2.5% as calculated from five assays performed on the same plasma on 10 different occasions.

Inhibition of anti-factor VIII activity. The ability of F(\(\text{ab}'\))\(_2\) fragments from healthy donors and of IgG pools to inhibit anti-factor VIII autoantibody activity was assessed by incubating patients' plasma or F(\(\text{ab}'\))\(_2\) fragments adjusted to an amount yielding an anti-factor VIII activity of 1.7 BU, with increasing amounts of the IgG or F(\(\text{ab}'\))\(_2\) fragments to be tested overnight at 4°C. Residual anti-factor VIII activity was then measured as described above. Based on the confidence limits of measurements of factor VIII activity, an increase by more than 10% of residual factor VIII activity in the reaction mixture containing patients' autoantibodies and test IgG or F(\(\text{ab}'\))\(_2\) as compared with residual factor VIII activity in a mixture of anti-factor VIII autoantibodies with phosphate-buffered saline (PBS) was considered as significant; an increase by 15% of residual factor VIII activity corresponded to 25% inhibition of anti-factor VIII activity by normal IgG and defined the threshold of positivity for the detection of anti-anti-factor VIII activity.

RESULTS

Anti-factor VIII autoantibodies in pools of normal IgG. Because natural anti-factor VIII antibodies are found in approximately 20% of healthy blood donors, we first investigated the presence of anti-factor VIII activity in pools of normal IgG. None of four therapeutic preparations of IVlg exhibited factor VIII-neutralizing activity when tested at final concentrations ranging between 0.18 and 12 mg/mL. No anti-factor VIII activity was observed in IgG from plasma pools of healthy males lacking natural anti-factor VIII activity in plasma and in pooled IgG from unselected multiparous women (tested at concentrations of 0.18 to 12 mg/mL). IgG from pooled plasma of multiparous women exhibited an anti-factor VIII activity of 1 BU when tested at the concentration of 12 mg/mL. Thus, the presence of individuals with natural anti-factor VIII antibodies among donors contributing to an IgG pool is not necessarily associated with the expression of anti-factor VIII activity in the pool.

Neutralizing antibodies against anti-factor VIII autoantibodies in pools of IgG. We compared IVlg (Sandoglobulin) that are prepared from plasma of unscreened donors with a pool of IgG from male donors selected for the lack of natural anti-factor VIII antibodies for the ability of these preparations to neutralize anti-factor VIII autoantibodies. Figure 1 shows the maximal inhibition of anti-factor VIII activity of autoantibodies from nine patients that was observed with IVlg and with the IgG pool from male donors. Maximal inhibition was observed at a specific molar ratio between anti-factor VIII antibodies and the source of neutralizing antibody within a large range of molar ratios between antibodies that we tested. No difference was found between IVlg and pooled IgG from selected male donors with regard to the frequency of inhibitory activity against anti-factor VIII activity of autoantibodies from nine patients with anti-factor VIII autoimmune disease (Fig 1).

Thus, removal of donors with natural anti-factor VIII antibodies did not enhance the inhibitory potential of pooled IgG against randomly selected anti-factor VIII autoantibodies. As shown in Fig 2, a higher inhibitory capacity towards anti-factor VIII autoantibodies was found in the pool of IgG from 500 multiparous women than in IVlg.

Relationship between the age of donors and expression in IgG of neutralizing activity against anti-factor VIII autoantibodies. We prepared F(\(\text{ab}'\))\(_2\) fragments of IgG from the serum of 13 healthy individuals between the ages of 20 and 30 years and 14 healthy donors between the ages of 50 and 63 years. The F(\(\text{ab}'\))\(_2\), preparations were then compared for their ability to inhibit anti-factor VIII autoantibody activity. Target autoantibodies were F(\(\text{ab}'\))\(_2\) fragments prepared from the plasma of three patients with anti-factor VIII autoimmune disease. A twofold higher frequency of neutralizing activity against the autoantibodies was observed with
IgG of aged donors as compared with IgG of younger donors (Fig 3 and Table 1).

Synergistic effect of pooled IgG on the expression of neutralizing activity against anti-factor VIII autoantibodies. We investigated the ability of F(ab')2 fragments of IgG from four young donors (donors 2 through 5 of Fig 3) and of various combinations of these F(ab')2 fragments to neutralize anti-factor VIII activity of patients' autoantibodies. Combinations were equimolar mixtures of antibodies. Anti-factor VIII autoantibodies from three patients were used as idiotypic targets. As shown in Fig 3, none of the F(ab')2 fragments exhibited neutralizing activity against any of the autoantibodies on their own. Mixing F(ab')2 fragments from two of the donors resulted in no significant expression of anti-factor VIII neutralizing activity. However, mixtures of F(ab')2 fragments from three or four of the healthy donors resulted, for some of the combinations, in the expression of neutralizing activity against the autoantibodies from two patients but not against the autoantibodies from a third patient (Fig 4). A neutralizing activity against anti-factor VIII autoantibodies was also observed with a combination of F(ab')2 fragments of IgG from two aged donors, whereas none of the individually tested F(ab')2 fragments from these donors exhibited inhibitory activity.

![Fig 3](image1.png)

**Fig 3.** Neutralizing activity against anti-factor VIII autoantibodies of IgG from young (left panels) and aged (right panels) healthy male donors. The donors are identified by numbers on the abscissa. F(ab')2 fragments of IgG prepared from donors' serum (1.6 to 6.0 mg/mL) were tested for their ability to neutralize anti-factor VIII activity in F(ab')2 fragments of IgG of patients with anti-factor VIII autoimmune disease, as described in the legend to Fig 1. Target anti-factor VIII F(ab')2 fragments were adjusted to an amount yielding an anti-factor VIII activity of 1.7 BU. (Upper panels) Anti-factor VIII F(ab')2 fragments from patient Sal. (Middle panels) Anti-factor VIII F(ab')2 fragments from patient Cer. (Lower panels) Anti-factor VIII F(ab')2 fragments from patient Bes. The ordinate indicates maximal inhibition of anti-factor VIII activity that was observed. Optimal inhibitory molar ratios between patients' F(ab')2 and donors' F(ab')2 ranged between 0.53 and 15.80. Twenty-five percent inhibition of anti-factor VIII activity defined the threshold of positivity for anti-anti-factor VIII activity.

![Fig 4](image2.png)

**Fig 4.** Neutralizing activity against anti-factor VIII autoantibodies of combinations of IgG from four young healthy donors. Numbers on the abscissa identify the donors (same legend as in Fig 3). Combinations were equimolar mixtures of antibodies. Combinations of F(ab')2 fragments of IgG from healthy donors (0.125 to 0.500 mg/mL) were tested for their ability to inhibit anti-factor VIII activity in F(ab')2 fragments of IgG of patients with anti-factor VIII autoimmune disease, as described in the legend to Fig 3. The ordinate indicates maximal inhibition of anti-factor VIII activity that was observed.

**Table 1. Frequency of Anti-factor VIII Neutralizing Activity in IgG From Healthy Donors**

<table>
<thead>
<tr>
<th>Target F(ab')2</th>
<th>Anti-factor VIII Antibodies</th>
<th>Mean Frequency of Anti-factor VIII Neutralizing Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>F(ab')2 of IgG from young male donors (n = 13)</td>
<td>Sal. Cer. Bes.</td>
<td>0.23 0.38 0.38</td>
</tr>
<tr>
<td>F(ab')2 of IgG from aged male donors (n = 14)</td>
<td>0.64 0.71 0.78</td>
<td>0.70</td>
</tr>
<tr>
<td>F(ab')2 of IgG from nulliparous women (n = 7)</td>
<td>0.43 0.71 0.14</td>
<td>0.43</td>
</tr>
<tr>
<td>F(ab')2 of IgG from multiparous women (n = 8)</td>
<td>0.00 0.50 0.62</td>
<td>0.37</td>
</tr>
</tbody>
</table>

The frequency of anti-factor VIII neutralizing activity was calculated by dividing the number of donors of whom IgG exhibited neutralizing activity by the total number of donors tested.

*Target anti-factor VIII antibodies are identified by the patients' initials.
against anti-factor VIII autoantibodies (Fig 5). Thus, mixing IgG from as few as two or three donors may be sufficient for the expression by a pool of IgG of neutralizing activity against anti-factor VIII antibodies.

Neutralizing activity against anti-factor VIII autoantibodies of IgG from multiparous and nulliparous women. F(ab')\(_2\) fragments prepared from IgG of seven nulliparous women and eight multiparous women were compared for their ability to neutralize anti-factor VIII activity of autoantibodies from three patients. No difference in the frequency of occurrence of anti-factor VIII neutralizing activity was observed between the two groups of donors (Fig 6) or between age-matched female and male donors (Table 1).

DISCUSSION

Intravenous immunoglobulins for therapeutic use (IVIg) are preparations of IgG obtained from the plasma of over 15,000 healthy donors. IVIg contain a wide spectrum of natural IgG autoantibodies and complementary natural anti-idiotypic antibodies that are normally present in the serum of healthy individuals. Previous evidence from our laboratory indicates that IVIg contain anti-idiotypic antibodies against anti-factor VIII autoantibodies; thus, F(ab')\(_2\) fragments from IVIg inhibit anti-factor VIII activity in F(ab')\(_2\) fragments from patients' IgG. Sepharose-bound F(ab')\(_2\) fragments from IVIg specifically retain anti-factor VIII activity on affinity chromatography of F(ab')\(_2\) fragments from patients' IgG containing anti-factor VIII autoantibodies, and IVIg recognize on anti-factor VIII autoantibodies a paratope-related idiotope defined by a mouse monoclonal anti-idiotypic antibody. In the present study, we have investigated the contribution of selected groups of donors to the anti-idiotypic activity of pools of IgG against pathogenic autoantibodies. We have used autoantibodies from patients with anti-factor VIII autoimmune disease as targets for neutralization by pooled IgG because the studies mentioned above have demonstrated that inhibition of functional activity of these antibodies by IVIg is mediated by idiotypic interactions.

We have recently observed that the plasma of approximately 20% of randomly screened healthy blood donors contains natural IgG anti-factor VIII antibodies. However, IVIg do not express anti-factor VIII activity. It is possible that natural anti-factor VIII antibodies in IVIg are neutralized by anti-idiotypic antibodies and that the presence of donors with anti-factor VIII antibodies may interfere with expression of anti-idiotypic activity against anti-factor VIII autoantibodies in pools of IgG. No difference in the frequency of neutralizing activity against anti-factor VIII autoantibodies was observed between IVIg and a pool of IgG that had been prepared from donors selected on the basis of their lack of natural anti-factor VIII antibodies in plasma. These results may indicate that autoantibodies from patients who served as targets for neutralization assays express paratope-related idiotypes which are different from those that are expressed by natural anti-factor VIII antibodies.

Women represent one third of the donors of IgG in Sandoglobulin. Because pregnancy is associated with alloimmunization against fetal antigens and with a higher frequency of occurrence of certain autoimmune diseases, including anti-factor VIII autoimmune disease, we compared the inhibitory activity of IVIg with that of pooled IgG.
from 500 multiparous women. A higher frequency of neutralizing antibodies to anti-factor VIII autoantibodies was found in the pool of multiparous women. However, IgG from individually tested multiparous donors did not exhibit a higher frequency of neutralizing activity against anti-factor VIII antibodies than IgG from individually tested nulliparous donors or male donors. Taken together, these observations indicate that the exclusion of donors with natural anti-factor VIII antibodies does not enhance anti-idiotypic activity of an IgG pool against anti-factor VIII autoantibodies; in addition, pools of IgG from multiparous women exhibit more frequently a neutralizing activity against anti-factor VIII autoantibodies than pools from random donors. The results also suggest that pooling IgG from multiple donors results in the expression of an anti-idiotypic activity that cannot be predicted from that expressed in the IgG of each of the donors contributing to the pool.

Row and Tankersley have shown that IVIg contain V region-dependent dimers, of which the amount is related to the number of donors contributing to the pool. The probability of finding anti-idiotypic antibodies directed against a specific autoantibody increases with the number of donors contributing to the pool. However, it may be argued that an anti-idotype contributed by a particular donor would be subject to a high degree of dilution in the pool. The experiments depicted in Figs 4 and 5 indicate that pooling IgG from as few as two or three donors may be sufficient for the expression of a neutralizing activity against anti-factor VIII antibodies that was not detected in IgG of single individuals. Synergy between anti-idiotypic antibodies originating from donors in the pool is dependent on the broadening of the antibody repertoire with the increase in the number of donors contributing to the pool. The results emphasize the synergistic effect of pooling IgG, and the potential advantage of large pools of IgG with regard to the expression of anti-idiotypic activity against autoantibodies.

To investigate whether the age of the donors influences the expression of anti-idiotypic activity in IVIg, we compared the frequency of occurrence of neutralizing activity against disease-associated anti-factor VIII autoantibodies in IgG from young and aged healthy male donors. Anti-idiotypic antibodies against a variety of disease-associated autoantibodies have previously been found in IgG from healthy individuals. The higher frequency of anti-idiotypic antibodies against anti-factor VIII autoantibodies that we found in IgG of aged donors may be caused by an increase in the spectrum of the naturally expressed anti-idiotypic repertoire with aging and/or a decrease of antibody affinity and subsequent increase in multireactivity of antibodies in aged individuals. An increase in the ability to produce auto-anti-idiotypes during the immune response has been described in aging mice. It may be speculated that the increased expression of anti-idiotypes against pathogenic autoantibodies in aged individuals contributes to the lower frequency of incidence of certain autoimmune diseases with aging, despite the apparent loosening in the control expression of the autoimmune repertoire that is manifested by an increased frequency of occurrence of natural autoantibodies in serum.

Our observations suggest that aged donors and multiparous women may be privileged sources of anti-idiotypes in IVIg. Further studies are required to identify other categories of donors that would represent improved sources of IgG for therapeutic pools.

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REFERENCES

15. van Doorn PA, Rossi F, Brand A, Van Lint M, Vermeulen


