Inactivation of Factor VIII Coagulant Activity by Two Different Types of Human Antibodies

By Maria S. Gawryl and Leon W. Hoyer

Human antibodies that inactivate factor VIII procoagulant activity (VIII:C) are heterogeneous in their kinetic properties. We report here the properties of four type I and four type II antibodies classified according to Biggs et al. Type I antibodies have second-order inactivation kinetics and completely destroy VIII:C when present in high concentration; type II antibodies have more complex kinetics and do not completely inactivate VIII:C even when tested undiluted. The latter properties correspond to the in vivo finding in some patients that there is detectable VIII:C, even though there is also a significant inhibitor titer. It has been suggested that the antibody–antigen complex in these patients retains some VIII:C activity. This is unlikely, however, since protein-A-Sepharose (PAS) did not adsorb any VIII:C activity from mixtures of type II antibodies with normal human plasma. An alternate possibility, reduced VIII:C inactivation due to a steric effect of the factor-VIII-related protein (VIIIr, von Willebrand factor), appears to be a more important factor, since three of four type II antibodies had inactivating properties like type I antibodies when they were tested with separated VIII:C instead of plasma. Although the fourth type II antibody did not completely inactivate separated VIII:C, the residual coagulant activity was adsorbed from this mixture by PAS. These data indicate that type II anti-VIII:C react with different antigenic determinants than type I antibodies and that these determinants are partially blocked in the factor VIII complex by VIIIr.

Materials and Methods

Factor VIII Measurements

Factor VIII procoagulant activity (VIII:C) was measured by a one-stage method using factor-VIII-deficient human plasma as substrate. Factor VIII procoagulant antigen (VIII:CAg) was measured by an immunoradiometric assay using 125I-labeled Fab' prepared from a type I human anti-VIII:C plasma. Factor-VIII-related antigen (VIIIIR:Ag) was determined by an immunoradiometric assay using a rabbit antibody. The standard (1 U/ml) for all factor VIII measurements was pooled normal human plasma, prepared as previously described.

Anti-VIII:C Measurements

Inhibition of VIII:C procoagulant activity was determined by incubating equal volumes of pooled normal human plasma or separated VIII:C17 with a dilution of antibody plasma for 2 hr at 37°C. The residual VIII:C activity was then measured and in some studies the antibody titer was expressed in Bethesda units. This value was the reciprocal of the antibody plasma dilution that inactivated 50% of the VIII:C activity during the 2-hr incubation. The value for each antibody plasma was the mean of assays done at five different plasma dilutions.

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Supported in part by Research Grants HL 16626 and HL 16872 from the National Heart, Lung and Blood Institute and Training Grant AI 07080 from the National Institute of Allergy and Infectious Diseases.

Submitted April 15, 1982; accepted June 14, 1982.

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0006-4971/82/6005-0007$01.00/0
**Human Anti-VIII:C**

Eight antibody plasmas that inactivated VIII:C were studied in detail. One type I antibody was obtained from a patient with no previous hemostatic disorder (Ab1). The other three type I antibody plasmas were obtained from patients with severe classic hemophilia who had been repeatedly transfused (Ab2–4). All of the four type II antibodies (Ab5–8) occurred as autoantibodies. These plasma samples had been stored at −70°C for 0.5–12 yr before these studies. The inhibitor plasmas were obtained through the helpful cooperation of Drs. E. G. D. Tuddenham, J. Miller, and H. S. Weiss. One plasma (Ab8) was purchased from George King Biomedical, Inc. (Overland Park, Kans.).

The classification of antibodies as type I or type II followed the criteria of Biggs and coworkers. The relationship of residual VIII:C activity (logarithmic scale) to antibody concentration was determined after a 2-hr incubation with normal plasma at 37°C.

**Adsorption of Antibodies and Immune Complexes With Protein-A-Sepharose**

Antibody and VIII:C mixtures were adsorbed with protein-A-Sepharose (PAS) (Pharmacia Fine Chemicals, Piscataway, N.J.) after a 2-hr incubation at 37°C. Excess PAS (3 ml of a 20% suspension of PAS beads in saline) was added to 0.6 ml of the mixture and the incubation continued at 37°C for 15 min. The PAS beads were then removed by centrifugation and the supernatant fluid examined for residual VIII:C. The maximum IgG/PAS ratio in these experiments (10 mg IgG/ml PAS) was well below the capacity of the beads.

In some experiments, human anti-VIII:C antibodies were immobilized by adsorption to PAS before being mixed with VIII:C. After the beads had been incubated with the antibody-containing plasma for 2 hr at room temperature, the beads were washed 3 times with large volumes of barbital-buffered saline (0.125 M NaCl, 0.015 M barbital, 0.010 M sodium barbital, pH 7.5) (BBS). The supernatant fluid was examined in each experiment and it contained less than 2% of the anti-VIII:C activity.

The volume of beads was kept constant in these experiments by employing mixtures of antibody–PAS beads and untreated Sepharose 4B–CL. Undiluted normal human plasma or partially purified VIII:C was incubated with an equal volume of the antibody–beads for 2 hr at 37°C and the residual VIII:C was determined in the supernatant fluid after the beads had been removed by centrifugation. PAS beads saturated with normal human plasma IgG served as a control reagent for these studies.

The amount of anti-VIII:C adsorbed to PAS beads was calculated with the assumption that all plasma antibody was bound. This assumption was verified in several studies in which the adsorbed IgG was eluted from washed PAS–antibody beads at pH 2.4. A glycine-NaCl buffer (0.05 M glycine, 0.1 M NaCl, 0.02% sodium azide) was used at a buffer:bead ratio of 8:1 (v/v), the beads removed by centrifugation (2800 g) for 20 min at room temperature, and the supernatant fluid added to 1/40 volume borate buffer (0.1 M boric acid, 0.05 M sodium borate, 0.075 M sodium chloride pH 8.4). After dialysis against at least 250 volumes of borate-buffered saline, pH 7.5 (0.036 M boric acid; 0.005 M sodium hydroxide, 0.159 M sodium chloride), the eluate was concentrated to 1 ml by negative pressure ultrafiltration.

The amount of IgG eluted was determined by Laurell immunoelectrophoresis using rabbit antibodies specific for human gamma...
Plasma incubated with Antibody 100 Viii:C incubated with Antibody 1
Plasma plus Antibody 100 Viii:C plus Antibody 1
PAS Adsorption

C0 C0 C0

> 0
> 0
> 0

<1

Fig. 3. Viii:C inactivation by antibody 1. Dilutions of this type I antibody in 0.3 ml saline were tested with 0.3 ml normal human plasma (●) or with separated Viii:C (○). In parallel experiments, 0.6 ml PAS was added to similar mixtures after the initial 2-hr incubation. The residual Viii:C activity was then determined after the PAS beads had been removed by centrifugation from mixtures of Ab1 with normal human plasma (●) or with separated Viii:C (○). Similar patterns were identified using Ab2, 3, and 4.

Fig. 4. Viii:C inactivation by antibody 8. Dilutions of this type II antibody in 0.3 ml saline were tested with 0.3 ml normal human plasma (●) or with separated Viii:C (○). In parallel experiments, 0.6 ml PAS was added to similar mixtures after the initial 2-hr incubation. The residual Viii:C activity was then determined after the PAS beads had been removed by centrifugation from mixtures of Ab8 with normal human plasma (●) or with separated Viii:C (○). Similar patterns were identified using Ab6 and 7.

Table 1. Properties of Human Anti-Viii:C

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Titer (Bethesda Units/ml)</th>
<th>Class*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Autoantibody</td>
<td>800</td>
<td>I</td>
</tr>
<tr>
<td>2</td>
<td>Hemophiliac</td>
<td>218</td>
<td>I</td>
</tr>
<tr>
<td>3</td>
<td>Hemophiliac</td>
<td>79</td>
<td>I</td>
</tr>
<tr>
<td>4</td>
<td>Hemophiliac</td>
<td>14</td>
<td>I</td>
</tr>
<tr>
<td>5</td>
<td>Autoantibody</td>
<td>60</td>
<td>II</td>
</tr>
<tr>
<td>6</td>
<td>Autoantibody</td>
<td>59</td>
<td>II</td>
</tr>
<tr>
<td>7</td>
<td>Autoantibody</td>
<td>302</td>
<td>II</td>
</tr>
<tr>
<td>8</td>
<td>Autoantibody</td>
<td>102</td>
<td>II</td>
</tr>
</tbody>
</table>

*Classification by reaction kinetics and degree of Viii:C inhibition.4,5

Heavy chains.15 The anti-Viii:C titer of the eluted IgG was determined in the same way as the plasma samples.14

RESULTS

The Viii:C inactivating properties of 8 human antibodies were characterized by the method of Biggs and coworkers.5,6 Type I antibodies (Ab1-4), at high concentrations, inactivated more than 98% of the Viii:C in a manner consistent with second-order kinetics, resulting in a linear inactivation response (Fig. 1). Undiluted type II antibodies (Ab5-8) did not completely inactivate plasma Viii:C, and the Viii:C inactivation graph had a curvilinear pattern (Fig. 2). The source, titer, and inactivation patterns of the 8 antibodies are given in Table 1.

The basis for nonlinear inactivation by type II antibodies was investigated by incubating plasma–antibody mixtures with protein-A-Sepharose (PAS) to remove most IgG and any immune complexes formed by IgG1, IgG2, or IgG4 antibodies. Preliminary experiments established that all of the anti-Viii:C activity was adsorbed from the inhibitor plasma when a sufficient quantity of PAS was added.

In control studies, the adsorption of type I antibody–plasma mixtures with PAS had minimal effect on Viii:C inactivation (Fig. 3). Similarly, additional Viii:C inactivation was not noted when type II antibody–plasma mixtures were adsorbed with PAS. Typical data are given in Fig. 4 (Ab8) and Fig. 5 (Ab5). Thus, the nonlinear and incomplete Viii:C-inactivating characteristics of type II anti-Viii:C seen when type II antibodies are incubated with plasma cannot be attributed to the formation of immune complexes that retain Viii:C activity.

The potential role of another factor, steric interference by the factor-Viii-related protein (VIIIIR, von
Willebrand factor), was also considered. In these studies, type I and type II anti-VIII:C were tested with partially purified VIII:C that had VIII:C to VIIIIR:Ag ratios greater than 990:1—in contrast to the 1:1 ratio (by definition) in normal plasma.13

Type I antibodies had similar properties when tested with separated VIII:C, and the inactivating capacity was only slightly greater than that observed with intact plasma (Fig. 3). Subsequent adsorption of the antibody–VIII:C mixture with PAS had no further effect on the amount of residual VIII:C. Thus, VIIIIR did not affect the VIII:C-inactivating properties of the 4 type I antibodies.

In contrast, three of the type II antibodies (Ab6–8) inactivated much more VIII:C when it had been separated from VIIIIR (Fig. 4). No further augmentation of antibody potency was observed in these experiments if the antibody–VIII:C mixture was adsorbed with PAS. The other type II antibody, Ab5, retained type II characteristics when tested with separated VIII:C, and its properties were unchanged from those observed with whole plasma (Fig. 5). The adsorption of immune complexes by PAS removed VIII:C activity in this case, however. Thus, VIIIIR inhibited VIII:C binding by each of the four type II antibodies. In three cases the antibodies had type I properties when tested with separated VIII:C; in the fourth case (Ab5), the interaction produced an immune complex that retained VIII:C activity.

VIII:C Inactivation by Immobilized Antibodies

A second group of experiments were carried out with immobilized type I and type II antibodies. The quantity of type I or type II antibody plasma incubated with PAS was chosen so that there would be approximately 100 Bethesda units of anti-VIII:C adsorbed by each milliliter of PAS, and the amount of bound antibody was verified in each case by testing the supernatant fluid. In control experiments, normal human plasma IgG was adsorbed with PAS in the same way.

Immobilized type I anti-VIII:C had the same properties as did the antibody in solution. Both plasma VIII:C and separated VIII:C were inactivated—presumably by removal from solution—and the dose–response pattern was linear (Fig. 6). In contrast, the four type II antibodies adsorbed less VIII:C from plasma when they were bound to PAS (Fig. 7). The immobilized type II anti-VIII:C were potentially reactive, however, for they removed over 98% of the VIII:C activity when incubated with separated VIII:C. This pattern—reduced reactivity with plasma VIII:C and increased reactivity with separated VIII:C—was consistent for each of the four immobilized type II anti-
bodies. As expected, normal human IgG bound to PAS had no effect on either plasma or separated VIII:C, and 95% ± 7% (1 SD) residual activity was measured in three studies.

Both type I and type II antibodies could be eluted from the PAS with glycine-buffered saline, pH 2.5. Measurement of anti-VIII:C activity recovered in this way verified the calculated amount of antibody that had been immobilized.

The studies with immobilized type II antibodies strongly suggested that VIIIIR partially blocks the interaction of type II anti-VIII:C with VIII:C determinants. This conclusion was supported by the demonstration that VIIIIR in hemophilic plasma inhibited in a dose-dependent manner the inactivation of separated VIII:C by immobilized type II antibodies (Fig. 8). Hemophilic plasma VIIIIR had no effect on the properties of an immobilized type I antibody (Ab1, Fig. 8).

The Effect of Type I and Type II Anti-VIII:C on VIII:C:Ag and VIIIIR:Ag Measurements

Residual VIII:C:Ag and VIIIIR:Ag were measured in each of the studies described above. The residual VIII:C:Ag levels were similar to most of the VIII:C values, but higher values were noted after some adsorptions. Representative data for a type I antibody (Ab1) and a type II antibody (Ab6) are given in Tables 2 and 3. The immobilized type I and type II antibodies did not remove any VIIIIR from plasma (Tables 2 and 3) and the residual VIIIIR:Ag content in 10 separate experiments was 97% ± 4% (1 SD) of that in plasmas incubated with control beads. The separated VIII:C
Table 2. Protein-A-Sepharose Adsorption of Anti-VIII:C Incubated With Plasma or Separated VIII:C

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Volume* (μl)</th>
<th>VIII:C (U/ml)</th>
<th>VIII:Ag (U/ml)</th>
<th>VIII:Ag (U/ml)</th>
<th>VIII:Ag (U/ml)</th>
</tr>
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<tbody>
<tr>
<td>Ab1</td>
<td>1.5</td>
<td>0.04</td>
<td>0.05</td>
<td>0.48</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>0.21</td>
<td>0.32</td>
<td>0.37</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>0.38</td>
<td>0.51</td>
<td>0.30</td>
<td>0.37</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ab6</td>
<td>150</td>
<td>0.03</td>
<td>0.15</td>
<td>0.48</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.13</td>
<td>0.28</td>
<td>0.49</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>0.47</td>
<td>0.64</td>
<td>0.47</td>
<td>0.14</td>
</tr>
<tr>
<td>Buffer</td>
<td>300</td>
<td>0.48</td>
<td>0.55</td>
<td>0.50</td>
<td>0.44</td>
</tr>
</tbody>
</table>

*Volume of antibody plasma in total volume of 300 μl. To this was added 300 μl of either normal plasma or separated VIII:C (1.0 U/ml). The mixture was incubated for 2 hr at 37°C and the IgG adsorbed with 600 μl Protein-A-Sepharose.

†As each VIII:C source was diluted 1:2 with either dilute antibody plasma or buffer, 0.5 U/ml indicates no loss or inactivation.

had very little VIIIIR:Ag (<0.1 U/ml) prior to the adsorption.

**DISCUSSION**

The inactivation properties of type I and type II human anti-VIII:C have been compared in this study so that the basis for the distinction could be clarified. By studying the ability of protein-A-sepharose to remove residual VIII:C from solutions containing antigen–antibody complexes, we were able to show that four type II antibodies do not form immune complexes that retain VIII:C activity when they are tested with normal human plasma. Similar inactivation data and residual VIII:C values were obtained before and after protein-A-sepharose adsorption of mixtures containing plasma and type II antibodies. If the type II antibodies were immobilized on protein-A-sepharose before being exposed to plasma, 10%–40% less plasma VIII:C was inactivated (Fig. 7).

In these studies, the less effective VIII:C inactivating properties of type II antibodies appeared to be due to steric inhibition by the VIIIIR present in factor VIII complexes. This conclusion was based on the observation that type II antibodies inactivated partially purified VIII:C—free of VIIIIR—in the same way as do type I antibodies incubated with plasma. Not all type II antibodies behaved identically, however, for one of them (Ab5) had the same characteristics when tested with separated VIII:C or with plasma (Fig. 5). All VIII:C was removed from the Ab5–VIII:C mixture by protein-A-sepharose, however, while the addition of PAS had no effect on Ab5–plasma mixtures. These results indicate that Ab5 reacts with VIII:C at a site different from that by the other type II antibodies. In the case of Ab5, the immune interaction is prevented by VIIIIR, but the antigen–antibody complex formed in the absence of VIIIIR retains VIII:C activity. Unless the complex is removed from solution, as by adsorption with PAS, Ab5 only inactivates part of the VIII:C activity.

The conclusion that type II antibodies recognize VIII:C antigens separate from the procoagulant site was supported by inhibition experiments in which VIIIIR was added back to separate VIII:C (Fig. 8). VIII:C inactivation of PAS–Ab8 was inhibited in a dose-dependent manner by hemophilic plasma.

We conclude that the different kinetic properties of the two kinds of human anti-VIII:C are due to the different kinds of antigenic determinants with which they react. Type I antibodies appear to interact with a group of antigenic determinants near the part of the molecule responsible for procoagulant activity. In contrast, type II antibodies recognize determinants remote from this region, and they are partially inhibited when

Table 3. The Effect of Immobilized Anti-VIII:C on Plasma and Separated VIII:C

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Volume* (μl)</th>
<th>VIII:C (U/ml)</th>
<th>VIII:Ag (U/ml)</th>
<th>VIII:Ag (U/ml)</th>
<th>VIII:Ag (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab1</td>
<td>10</td>
<td>&lt;0.01</td>
<td>0.11</td>
<td>1.15</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.16</td>
<td>0.64</td>
<td>1.13</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.87</td>
<td>0.72</td>
<td>1.11</td>
<td>0.1</td>
</tr>
<tr>
<td>Ab6</td>
<td>120</td>
<td>0.31</td>
<td>0.68</td>
<td>1.20</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.35</td>
<td>0.66</td>
<td>1.20</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.59</td>
<td>0.84</td>
<td>1.00</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>(normal plasma)</td>
<td>120</td>
<td>0.90</td>
<td>1.20</td>
<td>10</td>
</tr>
</tbody>
</table>

*Volume of antibody or control plasma adsorbed to 400 μl protein-A-Sepharose beads (see Methods). These beads were then washed and incubated with 400 μl of normal plasma or separated VIII:C (1.0 U/ml) for 2 hr at 37°C.

†The beads were removed from the mixtures by centrifugation and assay of the supernatant. In this table, 1.0 U/ml indicates no loss or inactivation.
VIII:C is associated with VIIIIR in the intact factor VIII complex. This interpretation is consistent with Green's observation that type I antibodies rapidly and completely inactivated the residual VIII:C activity that was left when plasma was incubated with type I antibodies.17

It is not certain why type II antibodies partially inhibit VIII:C of normal plasma or why the inactivation-concentration relationship is complex (Fig. 2). This pattern may indicate that there is heterogeneity in the antibody specificity so that some of the antibodies inactivate plasma VIII:C while other antibodies can only react with the separated coagulant protein. Alternatively, and more likely, the heterogeneity in plasma factor VIII may cause some VIII:C to be susceptible to inactivation while other VIII:C is protected by a close interaction with the VIIIIR.

Type II antibodies bound to PAS are even less effective in their ability to inactivate plasma VIII:C. In this case, there are potential steric effects produced by both VIIIIR and the protein-A-sepharose. As a result, the incomplete VIII:C inactivating properties of type II antibodies are exaggerated when they are bound to PAS (Fig. 7). Similar observations have been reported for rabbit anti-VIII:C immobilized by coupling to agarose.18 This steric effect was not detected with type I antibodies (Fig. 6).

Thus, the complex inactivating properties of type II antibodies are due to the antigenic determinants with which they react and the steric interference by the VIIIIR protein that partially shields the antigens. In addition, one type II antibody formed an immune complex that retained VIII:C activity. Only one of four type II antibodies had this property, however, and it was demonstrable only when the antibody was added to separated VIII:C. None of the type II antibodies formed VIII:C immune complexes which had residual coagulant activity when they were mixed with unfractionated plasma. For this reason, it is still not certain whether patients with type II antibodies retain some VIII:C activity in immune complexes or if they have, in vivo, a heterogeneous population of VIII:C molecules, some of which retain activity because they are protected by VIIIIR.

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Inactivation of factor VIII coagulant activity by two different types of human antibodies

MS Gawryl and LW Hoyer