Factor VIII Coagulant Antigen in Hemophilic Plasma: A Comparison of Five Alloantibodies

By Howard M. Reisner, William A. Price, Philip M. Blatt, Emily S. Barrow, and John B. Graham

Five independent alloantibodies directed to factor VIII coagulant antigen (VIII:CAg) were assessed against normal, von Willebrand's disease, and severe hemophilia A plasmas. Immunoradiometric assays (IRMAs) were developed for each antibody, one of which had arisen "spontaneously" and four in transfused hemophiliacs. The correlations between assays were very high for normal, vWd, and both CRM- and CRM+ hemophiliacs. This suggests that IRMAs may be developed from almost any reasonably high titered alloantibody and used with confidence in diagnosing CRM+ hemophilia A in utero by fetoscopy.

Alloantibodies (inhibitors) that arise in certain hemophilic and normal individuals can be neutralized in vitro by an antigen of normal plasma (VIII:CAg) closely related to coagulant factor VIII activity (VIII:C). Some hemophilia A plasmas (often designated as A-) lack VIII:C activity but contain VIII:CAg, and are thereby able to neutralize alloantibodies directed toward VIII:C. There is disagreement, however, as to whether all severe hemophiliacs or only a fraction of them have this functionally abnormal, immunologically detectable, cross-reacting material (CRM) in their plasma. This disagreement between studies might be due to differences in alloantibody specificity, or in technique, since antibody neutralization is a difficult test to interpret and is, at best, only semiquantitative.

The inadequacies of neutralization assays have stimulated the development of immunoradiometric assays (IRMAs) for VIII:CAg that allow for precise quantitation of this antigen in plasma. All reports to date agree that only a proportion of hemophiliacs have detectable VIII:CAg (i.e., are CRM+), but all such studies have relied on antibodies from single individuals. Hence, it is unclear whether anti-VIII:C antibodies are homogeneous in specificity. We have attempted to answer this question by examining the immunoreactivities of one spontaneous and four hemophilic alloantibodies to VIII:C against a panel of normal, von Willebrand's disease (vWd), and hemophilia A plasmas using IRMAs. In general, we found high, although not complete, correlation of test results between the antibodies.

Materials and Methods

Subjects

Ten members of the laboratory staff, known from history and pedigree to be normal, served as controls. A normal pool was prepared by mixing equal parts of plasma from each of the ten subjects. Plasmas from 43 male hemophilic subjects and 2 severely affected persons with vWd were obtained through the Hemophilia Center of North Carolina at Chapel Hill, and the Department of Hospital Laboratories of North Carolina Memorial Hospital, Chapel Hill, N.C.

Diagnosis was established by pedigree analysis and specific factor assay. Determination of bleeding time, factor-VIII-related antigen, and Willebrand factor levels was done as described elsewhere when necessary for the exclusion of von Willebrand's disease.

All blood samples were obtained under the rules of informed consent after approval by our local Human Investigations Committee and in accord with assurance filed and approved by the Department of Health, Education and Welfare.

Plasma Samples for Factor VIII Assays

Blood was collected from the antecubital vein with a plastic syringe using 1 part of 0.11 M sodium citrate to 9 parts of whole blood. Samples were centrifuged at 4000 rpm for 10 min at 6°C. Supernatant plasmas were aliquoted into polystyrene tubes and stored at −70°C for up to 30 mo. Samples were thawed once and discarded with the exception of several samples from hemophiliacs, which were available only in small quantity. VIII:C assays were done on either fresh samples (most hemophilic plasmas) or samples frozen at −70°C for not longer than 48 hr. It was shown in control experiments that freezing and thawing of normal samples up to 7 times did not affect VIII:CAg assay results. Repeat samples from 25 hemophilic individuals drawn at different times and stored for varying periods (about 1–30 mo) showed good reproducibility of VIII:CAg levels (see Results).

Factor VIII:C assays were performed using a standard one-stage activated PTT method with VIII:C-deficient plasma as substrate. Results are expressed in terms of the normal pool, which is assumed to have 100 U/dl (equivalent to 100%).

Factor VIII:C inhibition assays were carried out using the Bethesda method and are expressed in Bethesda units (BU).

Alloantibodies to VIII:C were obtained from an individual with acquired hemophilia (S-01) and from four individuals with the inherited disease (H-01 through H-04). Antibodies were selected on the basis of having a titer ≥ 1000 BU. Antibody-containing blood was collected in ACD, centrifuged at 3000 rpm for 30 min at 6°C, and the resulting plasmas were stored at −20°C. One of the antisera...
contamination of lgG preparations with plasmin. Isolation of

VIII:CAg immunoassay was carried out using an immunoradiometric technique described elsewhere in detail with the following modifications: IgG preparations from antibodies H-03 and H-04 were prepared using DEAE-Affigel Blue (BioRad) to prevent contamination of IgG preparations with plasmin. Isolation of immune complexes using antibodies S-01, H-01, 03, and 04 was accomplished using 125 VIII:C units of a commercial concentrate. Cryoprecipitate from a selected donor was used in preparation of antibody H-02. Yields of immunospecific 125I-anti-VIII:CAg are indicated in Table 1. All samples were diluted in standard assay buffer containing a 1/2 dilution of hemophilic canine plasma, the hemophilic canine plasma being substituted for semipurified human IgG as the carrier to reduce nonspecific binding and to slightly increase the assay sensitivity and reproducibility. Both carriers, however, produced similar VIII:CAg dose–response curves.

Most hemophilic samples were assayed at a single 1/2 dilution. Hemophilic samples with very low VIII:C levels and high VIII:CAg levels, however, were assayed at multiple dilutions, the same dose–response slope being obtained as with normal pool (for method of analysis see reference 13). Normal samples were generally assayed at dilutions of 1/4, 1/8, 1/16, 1/32. Sensitivity was calculated for each individual assay as the percent of normal pool, which was equivalent to nonspecific binding plus 2 standard deviations; samples with a lower binding level were considered to have a value of VIII:CAg that was undetectable.

RESULTS

Isolation of 125I-Anti-VIII:CAg Antibodies

Plasmas with high titered inhibitors of VIII:C activity (> 1000 BU) were chosen as a source of antibody (line 1, Table 1). All five plasmas proved suitable for assay of VIII:CAg. The yield of 125I-Fab' fragments recovered as immune complexes following Biogel A-5m chromatography ranged from 0.2% to 3% of total counts added to assay tube. The specificity of four of the isolated antibodies (H-01, H-02, H-03) was tested using a panel of normal, vWd, and hemophilic plasmas. (Antibody H-04, available only in limited amounts, was used primarily to assay hemophilic samples.) When aliquots of normal plasmas were assayed using the four antibody preparations, the degree of 125I-Fab' binding observed using each antibody was highly correlated (Table 2). The lowest correlation observed (between H-02 and H-03) was significant at the 1% level. Mean presumptive VIII:CAg values did not significantly differ from antibody to antibody and were significantly correlated to VIII:C values (r = 0.71, 0.82, 0.70, and 0.87 for S-01, H-01, H-02, and H-03, respectively). The mean value of VIII:C (118%) was, however, significantly higher than mean VIII:CAg levels in all cases (Table 2). The high degree of interimmunoassay correlation (correlation matrix, Table 2) and the significant correlation between VIII:C and VIII:CAg suggested that the four antibody preparations were, in fact, detecting VIII:CAg.

Plasma samples from two persons with severe homozygous vWd were available for assay. Levels of VIII:C, VIIIIR:Ag, and VIIIIR:WF were ≤ 1%.

Table 1. Alloantibodies to VIII:CAg

<table>
<thead>
<tr>
<th>Inhibitor titer (Bethesda units)</th>
<th>S-01</th>
<th>H-01</th>
<th>H-02</th>
<th>H-03</th>
<th>H-04</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-Fab' recovered (%)*</td>
<td>3.600</td>
<td>9.300</td>
<td>3.000</td>
<td>1.100</td>
<td>1.000</td>
</tr>
<tr>
<td>Binding at standard antigen concentration (%)†</td>
<td>0.28</td>
<td>0.34</td>
<td>0.24</td>
<td>0.54</td>
<td>0.74</td>
</tr>
<tr>
<td>Sensitivity (%) of normal pool‡</td>
<td>0.71</td>
<td>0.78</td>
<td>0.70</td>
<td>0.71</td>
<td>0.73</td>
</tr>
</tbody>
</table>

*Total counts 125I-Fab' recovered from the first peak of A-5 M column divided by the total counts applied to the column.
†125I-Fab' counts bound in the presence of a 1/2 dilution of normal pool plasma divided by the total counts added to assay tube.
‡Percent of normal pool that is equivalent to nonspecific binding (buffer blank) plus 2 standard deviations. Data represent the mean of at least 3 independent assays.

Table 2. Factor VIII Values in Normal Plasma

<table>
<thead>
<tr>
<th>VIII:CAg</th>
<th>VIII:C</th>
<th>S-01</th>
<th>H-01</th>
<th>H-02</th>
<th>H-03</th>
<th>H-04</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td></td>
<td>118.0</td>
<td>83.2</td>
<td>81.4</td>
<td>79.7</td>
<td>96.5</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>25.6</td>
<td>17.1</td>
<td>17.5</td>
<td>21.7</td>
<td>11.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Number of samples</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Pairwise Correlation Matrix (r values)

<table>
<thead>
<tr>
<th>Normal Plasma</th>
<th>H-01</th>
<th>H-02</th>
<th>H-03</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-01</td>
<td>0.92</td>
<td>0.96</td>
<td>0.87</td>
</tr>
<tr>
<td>H-01</td>
<td>—</td>
<td>0.95</td>
<td>0.86</td>
</tr>
<tr>
<td>H-02</td>
<td>—</td>
<td>—</td>
<td>0.79</td>
</tr>
</tbody>
</table>
VIII: CAg in Hemophilic Plasmas

Significant levels of VIII: CAg were found, however, in both samples with all antibodies tested (VIII: CAg range of 4.4%–7.0% and 5.2%–8.0%, respectively), although different plasma samples obtained previously from these individuals tested with antibody S-01 had not shown VIII: CAg. 12

VIII: CAg in Hemophilic Plasma

Samples of plasma from 43 hemophiliacs were selected from a larger group of affected subjects on the basis of reactivity with antibody H-01 (Table 3). An attempt was made to include samples with high VIII: CAg but very low VIII: C values. VIII: C values of all but 6 individuals were less than 1% of control, the latter 6 samples being in the 2%–3% range (column 1, Table 3). Samples 1–18 were clearly classified as CRM + by all antibodies and had VIII: CAg values in excess of VIII: C activity (columns 1–6, Table 3). Samples 19–43 had no detectable VIII: CAg activity with antibody H-01 and were classified as CRM +. Nine of these samples (36%) showed low levels of immunoreactivity (0 < VIII: CAg ≤ 2%) with at least one of the additional antibodies (columns 4–6, Table 3).

With the exceptions noted above and CRM + sample 14, there was very good correlation between VIII: CAg values obtained with the 5 antibodies. Mean values of VIII: CAg for each antibody were essentially the same (range of 6.5%–8.8%). The pairwise correlation matrix (Table 3) shows coefficients greater than 0.90 in all cases (samples 14a and b were omitted from these calculations). When sample 14 was first tested (14a), a considerable discrepancy was noted between H-02 and the 4 other antibodies. When retested using a fresh sample (14b), consistently higher values (VIII: CAg range of 68%–100%) were obtained (samples 14a and b, Table 3).

To measure assay reproducibility and biologic variability of VIII: CAg over a period of time, repeated samples from 25 hemophiliacs drawn on different dates were assayed with antibody H-01. Fifteen of those tested had no detectable VIII: CAg on any bleeding date. Ten individuals had detectable VIII: CAg on all bleeding dates. The mean coefficient of assay variation determined by 7 replicate determinations on dilutions of a single normal pooled plasma (1/12, 1/64, and 1/28 dilutions) was 0.15.

**DISCUSSION**

Some, but not all, severe hemophiliacs may be classed as CRM + using IRMAs specific for VIII: CAg. It has not been known, however, whether different alloantisera yield identical classification. The use of IRMAs in immunochemical studies of the VIII: C molecule, and in prenatal diagnosis of hemophilia A, 16 makes knowledge of the fine specificity of anti-VIII: CAg antibodies increasingly important.

Most high titered inhibitors to VIII: C appear to be usable for establishing IRMAs for VIII: CAg, since all five antisera tested in this study yielded sufficient 125I-Fab' fragments for use in assays. A potential problem is the presence of multiple antibody specificities in hemophilic plasmas, since commercial factor VIII concentrates used in isolating specific anti-VIII: CAg antibodies may allow additional specificities.
directed toward non factor-VIII-related plasma antigens to be isolated. We encountered this problem with antibody H-02, which contained both anti-VIII:C Ag and anti-Ag(x) (an unrelated low-density lipoprotein allotype). Specificity for VIII:CAg was achieved by a modification of the isolation procedure in which cryoprecipitate from an Ag(x−) individual was used (manuscript in preparation).

The specificity of the five antibodies for VIII:CAg is attested to by the following: (A) The low binding values in the plasma of two severe vWD subjects and hemophilic samples 19–43 (the CRM− group). (B) The high interassay correlation between normal and CRM+ hemophilic samples (values ranging from 0.79 to 0.99, Tables 2 and 3). (C) The significant correlations between VIII:C and VIII:CAg values in normal plasma (values ranging from 0.70 to 0.87, Table 2). (D) Antibody H-01 reacted with isolated procoagulant VIII produced by calcium dissociation17 but not with VIIIR:Ag (unpublished observations).

The significant difference between mean VIII:C and VIII:CAg values found in normal plasmas is unexplained but has been observed repeatedly. Possibly, the VIII:CAg in each individual expresses a narrower array of antigenic determinants than the VIII:CAg in a pool prepared from multiple individuals.

When 18 hemophilic plasmas having VIII:CAg antigen clearly measurable with H-01 were assayed with four additional antibodies, all 18 plasmas were consistently classified as CRM+. The highly significant correlation coefficients between the antibodies (minimum r = 0.91, p = 0.001) suggest that the 1 “spontaneous” and 4 hemophilic antibodies detect a closely related set of antigenic determinants on the inactive VIII:CAg molecules present in CRM+ hemophilic plasmas. The reduced levels of VIII:CAg seen in 17 of the 18 CRM+ hemophilic samples are unlikely to represent qualitative antigenic variation in the VIII:C molecule, both because of the parallelism in the dose–response curves compared to normal plasma, and because of the very high interassay correlation in VIII:CAg dose. Hence, there is most probably a quantitative reduction in the level of VIII:CAg in the plasma of most of the CRM+ hemophilic samples.

About one-third of the hemophilic samples in our set that were classified as CRM− with antibody H-01 showed low levels of immunoreactivity with at least one additional antibody. Samples from any single hemophilic drawn over a period of up to 30 mo were consistently CRM+ or CRM− when assayed with antibody H-01. Although the mean coefficient of variation determined in the low dose range of the assay (1/32–1/256 dilution of normal plasma) appeared to change from antibody to antibody (0.15 for H-01, 0.26 for H-02), it is most unlikely that irreproducibility of the assays in the low dose range is the sole cause of the observed interassay discrepancies. Since the levels of reactivity in these samples approach the minimum level of detection for the IRMAs, assay-to-assay variation in zero dose might also contribute to this discrepancy. For example, sample 20, which showed low levels of immunoreactivity with 3 of 5 antibodies, might contain low levels of VIII:CAg. Six of eight of the discrepant samples show reactivity with only one of the antibodies, however, and no single antibody was responsible for most of the discrepant reactivity. These differences probably represent very small amounts of non-VIII:CAg activity in the antibody preparations or dispersity in the VIII:CAg antigens detected by the antibodies. The low levels of reactivity in these samples make further study impractical.

Our results should give support to attempts to determine by fetoscopy whether a male fetus has hemophilia A. The method should work efficiently in all CRM− kindred and probably would be effective in those CRM+ kindred where VIII:CAg levels are markedly reduced. All high titered antibodies used in this study were useful in detecting VIII:CAg in fetal blood, and results should be consistent between laboratories using different antibodies. Finally, the difference in reactivity of sample 14a toward 4 of the 5 antibodies (presumably as a result of in vitro degradation) suggests that IRMAs using multiple antibodies may become a valuable adjunct in structural studies of this important procoagulant molecule.

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