Double-Antibody Radioimmunoassay for Factor VIII-Related Antigen

David Green and Nancy Reynolds

A plasma protein required for the support of ristocetin-induced platelet aggregation was isolated from anti-hemophilic factor concentrate and radiolabeled with $^{125}$I. A double-antibody radioimmunoassay was developed, with use of specific rabbit anti-VIII related antigen serum and goat anti-rabbit globulin. The assay is sensitive, reproducible, and technically simple to perform. Values obtained in normal subjects ranged from 0.65 to 1.53 units, similar to our normal range for VIII coagulant activity (0.67–1.43 units). However, normal or increased values of VIII-related antigen were observed in VIII coagulant-deficient hemophiliacs. Also, concentrations of VIII-related antigen significantly exceeded coagulant concentrations in several patients with liver disease or disseminated intravascular coagulation, or both. Of a broad selection of congenital coagulation disorders examined, only patients with von Willebrand's disease had decreased VIII-related antigen concentrations, and these corresponded to the lowered concentration of ristocetin cofactor in the patients. In three transfused patients, VIII-related antigen values correlated with the concentration of the cofactor. Our results suggest that the radioimmunoassay of VIII-related antigen is a simple and valuable adjunct in the study of patients with clotting abnormalities.

Additional Keyphrases: clotting abnormalities • ristocetin cofactor • von Willebrand's disease

It is now apparent that the central defect in von Willebrand's disease is a deficiency or qualitative abnormality of the factor VIII-related antigen (VIII:Ag)$^1$ (1). Alterations in VIII:Ag appear to account for the prolonged bleeding time, abnormal platelet adhesiveness, and reduced responsiveness of platelets in plasma of such patients to aggregation induced by ristocetin (2); ristocetin cofactor (VIII:RCoF) activity appears to be a major functional expression of VIII:Ag (3). Instability of factor VIII coagulant activity (VIII:C) in the absence of VIII:Ag may also account for the decreased concentration of VIII:C in patients with von Willebrand's disease (4). Thus, accurate, reproducible assays for the antigen are essential for the recognition and management of patients with von Willebrand's disease.

The most commonly used method for quantitating VIII:Ag is the electroimmunoassay (5, 6). Commercially available reagents may be used, and results are generally reproducible from laboratory to laboratory. However, the method is incapable of quantitating antigen concentrations of less than 6–10% of normal, and is not suited for assaying many samples.

These deficiencies in the electroimmunoassay method are not found in radioimmunoassay techniques, which can precisely measure antigen concentrations of 1% the normal value or less. In 1972, Hoyer (7) showed that complexes of VIII:Ag and radiolabeled rabbit antibody to VIII:Ag were insoluble in one-quarter saturated ammonium sulfate. The percentage of precipitated radioactivity was proportional to the quantity of antigen in the test sample. Subsequently, Counts (8) and Ruggeri et al. (9) described immunoradiometric assays (IRMA) of VIII:Ag. In these techniques, rabbit antibody to VIII:Ag is labeled with $^{125}$I, fixed to a solid phase, and used to extract and label VIII:Ag in the test sample.

The technique of double-antibody radioimmunoassay (RIA) is widely used to measure plasma constituents. The test sample is incubated with specific antibody (usually from another species), radiolabeled antigen is added, and a second antibody is used to precipitate the immune complexes. The amount of radioactivity appearing in the precipitates is inversely proportional to the extent the specific antibody is neutralized by antigen in the test sample. The RIA has the advantages of being well-standardized, technically simple to perform, and suitable for batch analysis and automation.

A brief report of the use of this method for the assay of VIII:Ag was published by Paulissen et al. in 1975 (10); in this paper, we present a detailed description of the method and our results in a large number of subjects with a variety of coagulation disorders.

Materials and Methods

Preparation of Radio-labeled VIII:Ag

VIII:Ag was isolated and labeled with $^{125}$I as follows: Antihemophilic factor concentrate ("Profilate"; Abbott...
Scientific Products, South Pasadena, Calif. 91030) was chromatographed on agarose (Sepharose 6B; Pharmacia Fine Chemicals, Piscataway, N. J. 08854), with phosphate-buffered isotonic saline as eluent (11). Ristocetin cofactor (VIII:RCoF) activity was monitored (12) as being representative of the functional activity of VIII:RCoF. Fractions containing VIII:RCoF activity were identified and rechromatographed on agarose, with 1 mol/liter NaCl as eluent. This step dissociates VIII: RCoF from VIII:C (13). The void volume material was then labeled with $^{125}$I by the peroxide–lactoperoxidase technique (14) as follows:

Two milliliters of the column fraction containing VIII:RCoF of the highest specific activity, 0.5 ml of 0.3 mmol/liter KI, and 400 $\mu$Ci of $^{125}$I (carrier-free; Cambridge Nuclear, Cambridge, Mass. 02139) were mixed with 0.1 ml of 0.1 mg/ml lactoperoxidase (Calbiochem, Los Angeles, Calif. 92206). Baseline measurements of VIII:RCoF activity and trichloroacetic acid-precipitable (protein-bound) radioactivity were recorded. The latter was tested by adding 0.01 ml of the incubation mixture to 1.0 ml of a 5 g/liter solution of albumin (“Buminate”; Hyland Lab., Costa Mesa, Calif. 92626) and producing a precipitate with 1.0 ml of trichloroacetic acid, 300 g/liter. Radiolabeling was initiated by adding 0.2 ml of hydrogen peroxide (1 mmol/liter) to the incubation mixture. This results in rapid iodination, which is complete within 1 min and is monitored by repeating the assay for trichloroacetic acid-precipitable radioactivity. The radiolabeled VIII:RCoF was separated from the lactoperoxidase and free $^{125}$I by further chromatography on an agarose column with use of a borate/albumin buffer (0.13 mol boric acid, 70 mmol of NaOH, and 5 g of albumin per liter; pH 8.3). The buffer is heated to 56 °C for 30 min to destroy possible contaminating factor VIII-related protein present in the albumin.

The final product retained full VIII:RCoF activity and VIII:RCoF when compared with unlabeled preparations. Autoradiographic studies of immunoelectrophoretic and electroimmunoassay plates disclosed a single arc or “rocket” when the labeled material was studied with crude rabbit anti-VIII:RCoF serum. Sucrose density-gradient ultracentrifugation and electrophoresis on polyacrylamide gel revealed that 96% of the labeled protein had a molecular weight in excess of 106. The remaining 4% was identified as radiiodinated lactoperoxidase. After reduction with 2-mercaptoethanol, the molecular weight of the labeled protein was $2.3 \times 10^{6}$, the molecular weight characteristic of the factor VIII subunit (15). The labeled VIII:RCoF was stored at 4 °C. After 30 days of storage, there was some dissociation of the label from the VIII:RCoF; this free $^{125}$I could be removed by dialysis without affecting the quality of the residual labeled antigen.

Preparation of Rabbit Anti-VIII:RCoF Serum

Rabbit anti-VIII:RCoF serum was prepared from VIII:RCoF isolated from the plasma of a hemophilic patient, as we have previously described (11). This anti-serum prevents the ristocetin-induced aggregation of normal platelet-rich plasma, inhibits platelet retention by glass-bead columns, and weakly inactivates VIII:C. Dilutions of this antiserum in normal rabbit serum diluted fivefold with borate albumin buffer were used for the radioimmunoassay. In subsequent work, commercially obtained antiserum (Nordic, Tilburg, The Netherlands; or Behringwerke, Somerville, N. J. 08876) performed equally well.

Preparation of Normal Pooled Plasma

Blood from 10 or more normal donors was collected in sodium citrate (38 g/liter) in the volume ratio of nine parts of blood to one part of citrate, and centrifuged (1500 × g, 30 min) to provide platelet-poor plasma. The plasma was pooled and stored at −20 °C in 1-ml aliquots.

Performance of the Double-antibody Radioimmunoassay

For radioimmunoassay, 0.1 ml of test sample, either undiluted or diluted in albumin buffer, was incubated with 0.1 ml of rabbit anti-VIII:RCoF serum for 4 h at room temperature in disposable glass tubes (10 × 75 mm; Scientific Products, McGaw Park, Ill. 60085). Then, 0.1 ml of labeled VIII:RCoF with a radioactivity of 105 to 106 counts per min was added, and the incubation continued for 16 h at 4 °C. Next, 0.15 ml of goat anti-rabbit serum (Antibodies, Inc., Davis, Calif. 95616) was added and the mixture permitted to stand at room temperature for 4 h. The tubes were centrifuged, the precipitates washed three times with saline, and the total radioactivity in the precipitates was recorded. Each run included several dilutions of pooled plasma as well as a saline control. The precipitated radioactivity in the pooled plasma samples was counted and the counts were used to plot a standard curve. Under the conditions we used, the values obtained with 0.1 ml of pooled plasma diluted $y_0$ to $y_0$ were near the midpoint of the standard curve. The VIII:RCoF of the test plasma was determined from the standard curve and the plasma dilution used. Samples suspected of having subnormal activity were examined undiluted. Samples were frequently tested in duplicate, and the same sample was re-examined on different days, to assess reproducibility of the assay.

Other assays, including measurements of the functional activity VIII:RCoF, VIII:C, platelet retention by glass beads, and platelet aggregation were performed as previously described from our laboratory (11).

Results

Standard Curve

In Figure 1, the percent concentration of pooled plasma is plotted vs. percent precipitated counts per min at several concentrations of rabbit anti-VIII:RCoF serum. A 1/50 dilution of the antiserum resulted in the precipitation of more than 70% of the radioactivity in the mixture; this declined to 50% when diluted pooled
plasma was added to the mixture. A 1/400 dilution of the antiserum showed a linear relation between the log of plasma concentration and the log of percentage of counts precipitated, and therefore this dilution of antiserum was used for all assays. Of interest was the observation that normal rabbit serum, without antibody, bound about 20% of the labeled material and that this binding was also inhibited by pooled plasma. It is unlikely that this was due to a lack of protein in the mixtures, because all tubes contained about 5 g of albumin per liter. More likely this nonspecific binding is due to labeled protein that is not bound by specific anti-VIII:Ag antibody (for example, labeled lactoperoxidase).

Reproducibility of the RIA Method, and Typical Assay Results

In two different assays of five plasma samples the average percent deviation from the mean was 21%. Duplicate determinations of VIIIIR:Ag included in the same run varied by less than 5%. The results of typical assays, including raw counts, percent of counts precipitated, and VIIIIR:Ag values in units per milliliter are shown in Table 1. Duplicate determinations were performed on three test samples, and serially diluted normal pooled plasma. Each tube originally contained about 10^4 counts per min of radioiodinated VIIIIR:Ag. The percent precipitated radioactivity in the samples of pooled plasma was plotted on log-log paper vs. concentration in plasma, and the VIIIIR:Ag values of the test samples were determined from this curve. These values were similar to VIII:RCoF and VIII:C in these samples. Samples were run in batches with as many as 80 tests in one batch.

VIIIIR:Ag Values in Normal Subjects, Hemophiliacs, and Patients with von Willebrand’s Disease

Figure 2 shows the distribution of values for VIIIIR:Ag concentration in 31 normal subjects, 11 hemophiliacs, and 10 patients with von Willebrand’s disease of various degrees of severity. Normal subjects had a mean value of VIIIIR:Ag of 1.09 units, with a standard deviation of 0.22, giving a range of 0.65–1.53 units for 95% of the population. The values for the 13 men in this group were not significantly different from those of the 19 women. The mean value for the 11 hemophiliacs examined was 1.26 units, which is higher than the normal mean (1.09) but within the normal range. These subjects had VIII:C concentrations ranging from <0.01 to 0.15 units/ml.

Multiple laboratory studies in 10 patients with von Willebrand’s disease are presented in Table 2. In general, there was a close correspondence among the values for VIII:C, VIII:RCoF, and VIIIIR:Ag in these subjects.

Table 1. Typical Values for VIIIIR:Ag (RIA) Compared with RCoF (Washed Platelets), VIII:C, and VIIIIR:Ag (the Last Performed by Electroimmunoassay Method)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Total cpm</th>
<th>Pptd. cpm</th>
<th>Pptd. cpm, %</th>
<th>VIIIIR:Ag (RIA)</th>
<th>RCoF (washed platelets)</th>
<th>VIII:C</th>
<th>VIIIIR:Ag*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test 1</td>
<td>1:20</td>
<td>11 833</td>
<td>5830</td>
<td>49.3</td>
<td>1.04</td>
<td>1.00</td>
<td>1.00</td>
<td>0.90</td>
</tr>
<tr>
<td>Duplicate</td>
<td>1:20</td>
<td>11 763</td>
<td>6028</td>
<td>51.2</td>
<td>0.92</td>
<td>1.00</td>
<td>1.00</td>
<td>0.90</td>
</tr>
<tr>
<td>Test 2</td>
<td>1:4</td>
<td>12 000</td>
<td>5287</td>
<td>43.9</td>
<td>0.31</td>
<td>32.3</td>
<td>1.00</td>
<td>0.90</td>
</tr>
<tr>
<td>Duplicate</td>
<td>1:4</td>
<td>11 953</td>
<td>3936</td>
<td>45.1</td>
<td>0.28</td>
<td>1.53</td>
<td>0.31</td>
<td>0.36</td>
</tr>
<tr>
<td>Test 3</td>
<td>1:40</td>
<td>11 934</td>
<td>5000</td>
<td>41.9</td>
<td>3.60</td>
<td>32.3</td>
<td>2.50</td>
<td>2.45</td>
</tr>
<tr>
<td>Duplicate</td>
<td>1:40</td>
<td>11 323</td>
<td>4900</td>
<td>43.3</td>
<td>3.20</td>
<td>1.53</td>
<td>2.50</td>
<td>2.45</td>
</tr>
<tr>
<td>Pooled plasma</td>
<td>none</td>
<td>11 631</td>
<td>3563</td>
<td>30.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>11 671</td>
<td>3770</td>
<td>32.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>11 306</td>
<td>4282</td>
<td>36.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>11 523</td>
<td>5564</td>
<td>48.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:40</td>
<td>11 953</td>
<td>7840</td>
<td>65.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>buffer</td>
<td>12 060</td>
<td>8942</td>
<td>74.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Kindly performed by N. van Tilburg-electroimmunoassay method.

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Fig. 1. Curves relating precipitated radioactivity to concentration in plasma, for various dilutions of rabbit anti-VIIIIR:Ag serum Each point (except for those at zero antiserum concentration) represents the mean ±SE of six determinations done on different days. At an antiserum dilution of 400-fold, the relationship was linear and covered the broadest range of values.
Table 2. Comparison of VIII:Ag Concentrations by RIA with Other Hemostatic Variables in 10 Patients with von Willebrand's Disease

<table>
<thead>
<tr>
<th>Case</th>
<th>Bleeding time, min</th>
<th>Platelet retention</th>
<th>VIII:C</th>
<th>RCoF (washed platelets)</th>
<th>VIII:Ag (electroimmunoassay)</th>
<th>VIII:Ag (RIA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt; 15</td>
<td>15</td>
<td>0.03</td>
<td>&lt;0.01</td>
<td>—</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>&gt; 15</td>
<td>20</td>
<td>0.01 (0.30)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.01 (&lt;0.01)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.06 (0.10)&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>0.02 (0.15)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>&gt; 15</td>
<td>85</td>
<td>0.03 (0.25)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.01 (0.03)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.06 (0.30)&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.03 (0.20)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>&gt; 15</td>
<td>33</td>
<td>0.03</td>
<td>&lt;0.01</td>
<td>—</td>
<td>0.03</td>
</tr>
<tr>
<td>5P</td>
<td>—</td>
<td>—</td>
<td>0.56</td>
<td>0.17</td>
<td>0.17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.22</td>
</tr>
<tr>
<td>5C</td>
<td>&gt; 15</td>
<td>—</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.01</td>
</tr>
<tr>
<td>6P</td>
<td>&gt; 15</td>
<td>—</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.02</td>
</tr>
<tr>
<td>6C</td>
<td>—</td>
<td>—</td>
<td>0.88</td>
<td>0.35</td>
<td>0.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.55</td>
</tr>
<tr>
<td>7P</td>
<td>9.5</td>
<td>66</td>
<td>0.54</td>
<td>0.25</td>
<td>—</td>
<td>0.27</td>
</tr>
<tr>
<td>7C</td>
<td>6.5</td>
<td>63</td>
<td>0.49</td>
<td>0.25</td>
<td>—</td>
<td>0.35</td>
</tr>
<tr>
<td>Normal range</td>
<td>2–6</td>
<td>70–100</td>
<td>0.67–1.43</td>
<td>0.50–1.50</td>
<td>0.45–1.55</td>
<td>0.65–1.53</td>
</tr>
</tbody>
</table>


Plasma from Case 1 was examined before and after the infusion of Profilute (10 units/kg body wt) by measuring the functional activity of VIII:RCoF and by the radioimmunoassay technique (Figure 3). Immediately after this infusion, both methods recorded an increase and subsequently showed similar rates of decline. However, 24 h after the infusion, when functional activity was negligible (<5%), 0.31 unit of antigen was still detected by the radioimmunoassay. Post-transfusion data for cases 2 and 3 are also shown in Table 2. In these patients, as in Case 1, VIII:Ag exceeded VIII:RCoF functional activity following transfusion.

VIII:Ag Values in Patients with Various Coagulation Defects (Table 3)

VIII:Ag values within the normal range were observed in patients with a broad variety of congenital defects, including one patient with thrombasthenia and one with congenital thrombopoietin deficiency. Increased concentrations were noted in patients with thrombocytopenia and in three patients with circulating...
anticoagulants. High concentrations of VIIIIR:Ag were also observed in patients with liver disease and disseminated intravascular coagulation, and in four patients (Cases B, C, E and F), values of VIIIIR:Ag considerably exceeded those of VIII:C.

Discussion

The double-antibody radioimmunoassay was technically simple to perform and gave reproducible results. No prior sample preparation was required, either plasma or serum could be examined, and only 0.1 ml of the specimen was required. An example of the usefulness of the assay became apparent when we were asked to study the three-day-old son of a patient with severe von Willebrand's disease and were sent 0.5 ml of clotted blood from a heel puncture. The VIIIIR:Ag concentration in this serum sample was within the normal range, excluding severe von Willebrand's disease in this young patient. We recently participated in a large cooperative study of von Willebrand's disease (European Working Party on Factor VIII-Related Antigens). In 32 patients, the results of the RIA closely correlated with the mean of electroimmunoassay values obtained by six different laboratories (r = 0.95). In contrast to the electroimmunoassay, the RIA was more sensitive (lower limits detected: 1% vs. 6–10%) and was readily adapted to the processing of large numbers of samples, either in duplicate or in triplicate.

The specificity of the RIA is attested to by the fact that only patients with von Willebrand's disease, as defined by independent criteria, had abnormally low values. Concentrations of VIIIIR:Ag in patients with a broad spectrum of other coagulation disorders had either normal or increased values. In patients with von Willebrand's disease, including some with borderline abnormalities and others with the severe syndrome, values of VIIIIR:Ag correlated with the other abnormal parameters of this disorder. After transfusion, VIII: RCoF activity and VIIIIR:Ag disappeared in parallel, but at all times studied, concentrations of antigen were higher than functional activity. Perhaps not all of the antigen is biologically active (18).

Concentrations of VIIIIR:Ag in hemophilic patients averaged slightly higher than normal, and patients with circulating inhibitors of VIII:C had the highest values. In patients with acquired coagulation disorders, including thrombocytopenia, circulating anticoagulants, liver disease, and disseminated intravascular coagulation, concentrations of VIIIIR:Ag were generally increased. The increased values observed are probably a consequence of the pathophysiologic effects of the underlying disorders.
We gratefully acknowledge the advice and encouragement of Drs. Jacob Pruzaansky and Ennio C. Rossi, and we thank Dr. J. J. Veltkamp for permission to study his patients (cases 5 and 6).

References