Rabbit Factor VIII: Identification of Size Heterogeneity

By Margaret E. Rick, D. Eugene Wampler, and Leon W. Hoyer

Two forms of rabbit factor VIII procoagulant activity, distinguishable by size on gel filtration and ultracentrifugation, have been identified in normal rabbit plasma. These studies have been carried out with citrate-anticoagulated rabbit plasma obtained by cardiac puncture. Two peaks of factor VIII activity were obtained on agarose gel chromatography, using physiologic ionic strength buffers: a high molecular weight peak eluting at the void volume and a second peak eluting with smaller plasma proteins. The presence of high and low molecular weight factor VIII activities was confirmed by sucrose density gradient centrifugation. The two peaks of factor VIII activity remained distinct when proteolytic inhibitors were added to the plasma and eluting buffers. Both the high and low molecular weight factor VIII procoagulant activities were inhibited by antibodies to human and rabbit factor VIII, and both were activated by thrombin. The identification of size heterogeneity of factor VIII in normal rabbit plasma, in the absence of any modification by ionic strength, may permit more satisfactory study of the relationship of factor VIII size to function.

Purified human factor VIII is a large glycoprotein with a mol wt of 1,200,000 daltons as determined by sedimentation equilibrium. Recent reports indicate that human factor VIII activity is associated with a lower molecular weight fraction when factor VIII is exposed to high ionic strength buffers, suggesting that only a small portion of the large molecule is necessary for procoagulant activity.

While examining the association of coagulation factors with rabbit platelets, Tangen and co-workers noted that rabbit factor VIII activity could be identified in two plasma fractions by gel chromatography. One fraction corresponded to a molecular weight greater than 2,000,000 daltons; the second was a lower molecular weight fraction that was not further characterized. Because of the similarity to the high and low molecular weight factor VIII activities seen in human plasmas, rabbit factor VIII was studied as a model system to investigate factor VIII size heterogeneity and to characterize partially the lower molecular weight rabbit factor VIII activity obtained in the absence of ionic-strength manipulation.

MATERIALS AND METHODS

Buffers. The barbital-saline buffer was prepared by adding 7.3 g NaCl (0.125 M), 2.76 g barbital (0.015 M), and 2.06 g Na barbital (0.010 M) to sufficient deionized water to make 1 liter; the pH was adjusted to 7.5. For agarose gel chromatography, the pH of this buffer was adjusted to 6.8 with HCl. The imidazole buffer used for preparation of sucrose density gradients contained...
syringes and centrifuged at 3000 g for 15 mm at 4°C. Phenylmethyl sulfonylfluoride (PMSF, 10^{-3} M final concentration) was added immediately after separation from red blood cells.

Protolytic inhibitors. In several experiments, proteolytic inhibitors (Sigma Chemical Co., St. Louis, Mo.) were added to the fresh blood samples: soybean trypsin inhibitor (4 μg/ml final concentration) and aprotinin (10 units/ml final concentration) were added to the polycarbonate collecting tubes; phenylmethyl sulfonylfluoride (PMSF, 10^{-3} M final concentration) was added immediately after separation from red blood cells.

Factor VIII activity measurement. The residual factor VIII activity was then determined and compared to that of a control sample in which barbital-saline buffer (for the human antibody) or nonimmune goat serum (for the goat antibody) was used instead of antibody. One unit of antibody was defined as the quantity that inactivated 75% of the factor VIII activity in the control sample. The goat anti-rabbit antibody titer was 2.5.

Agarose gel chromatography. Six per cent agarose (Biogel A-5m, BioRad, Richmond, Calif.) columns (1.5 x 30 cm and 1.5 and 58 cm) were equilibrated with barbital-saline buffer, pH 6.8, containing 0.02%, sodium azide and 0.5%, t-aminocaproic acid (EACA). One-, two-, or four-milliliter samples were applied, and upward-flow elution was maintained at 20 ml/hr at room temperature using a peristaltic pump. Two- or four-milliliter fractions were collected, and factor VIII assays were performed within 4 hr. In several experiments, the eluting buffer contained the proteolytic inhibitors at the concentrations noted above.

Fractions containing factor VIII activity were concentrated by adding an equal volume of 30%, polyethylene glycol (PEG 6000, Baker Chemical Co., Phillipsburg, N.J.) with stirring for 1 hr at 4°C. Precipitates were collected by centrifugation at 39,000 g for 20 min at 4°C and were resuspended in 1/10 their original volume in barbital-saline buffer, pH 7.5.

Sucrose density gradient centrifugation. Thirteen-milliliter continuous 10-40%, w/v sucrose gradients were prepared in imidazole-buffered saline (either 0.14 M or 1.0 M) pH 7.4, with 0.02% azide.

Fresh rabbit plasma (0.5 ml) or a fraction from gel filtration (1.0 ml) was applied, and centrifugation was carried out using an SW 40 rotor at 40,000 rpm (201,800 g) for 24 hr at 20°C. Fractions (0.95-1.0 ml) were collected by gravity flow after puncture of the bottom of the tubes. They were assayed for factor VIII at 1:6 dilution in barbital-saline buffer to reduce the sucrose concentration. Samples that had been centrifuged in 1.0 M NaCl were diluted 1:6 with deionized water prior to assay to reduce both the sucrose and NaCl concentrations.

Thrombin activation of factor VIII. Bovine thrombin (Parke Davis, South Hackensack, N.J.) was purified according to the method of Lundblad. Factor VIII concentrates were incubated with 1/20 volumes of an aqueous suspension of phospholipid (Gliddex, Central Soya, Chicago, Ill.; final concentration 0.05 mg/ml) and 1/20 volume purified thrombin (final concentration 0.21

Immune inactivation of factor VIII was accomplished using both a “spontaneous” human factor VIII inhibitor (human anti-human) which had been previously characterized, and a goat anti-rabbit factor VIII (kindly supplied by Dr. Jean Dodds, Albany, N.Y.). This antibody was made to rabbit factor VIII concentrate enriched in high molecular weight factor VIII activity. An equal volume of diluted antibody was mixed with the test material and incubated for 1 hr at 37°C. The residual factor VIII activity was then determined and compared to that of a control sample in which barbital-saline buffer (for the human antibody) or nonimmune goat serum (for the goat antibody) was used instead of antibody. One unit of antibody was defined as the quantity that inactivated 75% of the factor VIII activity in the control sample. The goat anti-rabbit antibody titer was 2.5.

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Factor VIII activity measurement. Factor VIII activity was measured by a one-stage assay using factor VIII-deficient substrate plasma (George King Bio-Medical, Inc., Salem, N.H.). One unit of factor VIII activity was defined as the activity present in 1 ml of pooled normal human plasma. In this system, factor VIII procoagulant activity from fresh normal rabbit plasma was approximately 4 units/ml, while that from frozen rabbit plasma varied from 2 to 3 units/ml.

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Agarose Gel Chromatography

The size of rabbit factor VIII was estimated by 6% agarose gel chromatography of fresh rabbit plasma (Fig. 1). The bulk of the factor VIII activity eluted in the void volume, but a second smaller peak of activity eluted later at the leading edge of the major plasma protein peak. The same pattern was seen in six other experiments, both with fresh and with frozen rabbit plasmas.

To test the possibility that two peaks of activity were due to proteolysis of factor VIII by plasma enzymes, rabbit blood was centrifuged at 4°C in the presence of soybean trypsin inhibitor and aprotinin, and PMSF was added to plasma immediately after separation from red cells. The plasma was chromatographed in the presence of these three proteolytic inhibitors with the resultant pattern shown in Fig. 2. Two peaks of factor VIII activity were again apparent.
with increased recovery of factor VIII in the second peak. Similar procoagulant recovery patterns were observed in five other experiments; the void volume absorbance (OD 280) varied from 0.02 to 0.14 units.

In order to determine if the apparent size differences of the two factor VIII activity peaks represented different molecular species as opposed to the products of an association–dissociation reaction, the peak fraction(s) from the high molecular weight and low molecular weight areas were each rechromatographed separately on 6% agarose using barbital-saline buffer. Each fraction appeared in the expected position, the high molecular weight factor VIII eluting at the void volume, and the low molecular weight factor VIII activity eluting at the beginning of the major plasma protein peak.

**Sucrose Density Gradient Centrifugation**

The presence of two sizes of rabbit factor VIII was also evaluated by a second, complementary technique, sucrose density gradient centrifugation. Fresh rabbit plasma was centrifuged at 20°C for 24 hr on two sucrose gradients, one of physiologic ionic strength (0.14 \( M \) NaCl) and the other of high ionic strength (1.0 \( M \) NaCl). As shown in Fig. 3, top panel, the physiologic ionic strength gradient revealed two peaks of factor VIII activity similar to that seen with the separation on 6% agarose. In contrast, when fresh rabbit plasma was centrifuged on 1.0 \( M \) NaCl sucrose gradients, the bulk of the factor VIII activity was shifted to a position of apparent intermediate size (Fig. 3, lower panel).
Table 1. Immunologic Inactivation of Factor VIII

<table>
<thead>
<tr>
<th>Factor VIII Source</th>
<th>Activity (Units/ml)</th>
<th>Antibody</th>
<th>Antibody Dilution</th>
<th>Factor VIII Activity Retained (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled normal human plasma</td>
<td>1.00</td>
<td>HAH</td>
<td>1:150</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>HAH</td>
<td>1:50</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>HAH</td>
<td>Undiluted</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Diluted rabbit plasma</td>
<td>1.00</td>
<td>HAH</td>
<td>1:50</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>HAH</td>
<td>Undiluted</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>GAR</td>
<td>Undiluted</td>
<td>10</td>
</tr>
</tbody>
</table>

Pooled normal human plasma or normal rabbit plasma was incubated for 1 hr at 37°C with a human anti-human factor VIII (HAH) or with a goat anti-rabbit factor VIII (GAR). Residual factor VIII activity was then measured.

Shoulder was detected in the same fractions as the light factor VII peak, but this could not be definitely separated from the major portion of the factor VIII activity. These findings were consistent in three separate experiments. Estimated sedimentation values of 4.9S for the low molecular weight and 10.9S for the high molecular weight factor VIII activities were calculated by comparison to the sedimentation of IgM, fibrinogen, and IgG.\(^\text{13}\)

**Immunologic Inactivation of Factor VIII Procoagulant**

Initial experiments were done to determine if a spontaneous human inhibitor to factor VIII would also inactivate rabbit factor VIII activity. As shown in Table 1, the human antibody inactivates rabbit factor VIII, though not to the extent that it inactivates human factor VIII. Similar results have been noted in studies with “species specific” antibodies directed against bovine as opposed to human factor VIII.\(^\text{1}\) Inactivation of normal rabbit plasma by the goat anti-rabbit factor VIII is shown for comparison.

![Fig. 4. Immunologic inactivation of high and low molecular weight factor VIII. Column fractions were concentrated by PEG precipitation prior to incubation with antibody for 1 hr at 37°C. The amount of antibody added in 2.0-ml total volume is indicated. The initial factor VIII activity of the high molecular weight concentrate was 0.31 units/ml, and the low molecular weight concentrate was 0.8 units/ml.](image)
The immune inactivation of polyethylene glycol concentrates of the high molecular weight and low molecular weight factor VIII activities from a 6% agarose column is shown in Fig. 4. Inactivation of both activities by an antibody to factor VIII is important to exclude the presence of a different procoagulant distinct from factor VIII. The inhibition of both activities by the goat anti-rabbit factor VIII occurs in a dose-response pattern, as indicated by the increasing inactivation when larger amounts of antibody are added.

**Thrombin Activation**

Figure 5 shows the thrombin activation curves for the high and low molecular weight factor VIII fractions from a 6% agarose column. Both fractions showed the characteristic activation followed by loss of procoagulant activity that is seen with factor VIII. Since the initial factor VIII activities of the high and low molecular weight fractions were different, a direct comparison of the magnitude of activation of each fraction could not be made.

**Effect of Dilution on Factor VIII Activity**

The low molecular weight factor VIII had a steeper slope for the dose-response curve (clotting time versus the logarithm of concentration) than either normal rabbit plasma or the separated high molecular weight factor VIII. Typical results are given in Fig. 6, and a summary of the data is given in Table 2. The steeper slope of the low molecular weight fractions makes comparisons of relative activity quite arbitrary; as a consequence, estimates of percent recovery from columns are inexact. The low molecular weight factor VIII
**Table 2. Effect of Dilution on Factor VIII Activity**

<table>
<thead>
<tr>
<th>Factor VIII Source</th>
<th>Increase in Clotting Time for Each Ten-fold Dilution*</th>
<th>No. of Determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled normal human plasma</td>
<td>20.3 ± 3.8</td>
<td>9</td>
</tr>
<tr>
<td>Normal rabbit plasma (single donor)</td>
<td>27.4 ± 3.8</td>
<td>16</td>
</tr>
<tr>
<td>Rabbit high molecular weight factor VIII</td>
<td>25.7 ± 4.6</td>
<td>14</td>
</tr>
<tr>
<td>Rabbit low molecular weight factor VIII</td>
<td>37.8 ± 5.3</td>
<td>9</td>
</tr>
</tbody>
</table>

*Mean ± 1 SD. The difference between the high molecular weight factor VIII and normal rabbit plasma was not significant (p > 0.2), while the differences between the low molecular weight factor VIII and normal rabbit plasma, or the low molecular weight and high molecular weight factor VIII were significant (p < 0.001). The difference between normal rabbit plasma and normal human plasma was also significant (p < 0.002). The statistical analysis was carried out using Student’s t test.

activity values that are given are estimates based on the clotting times for undiluted fractions.

**DISCUSSION**

The actual size of factor VIII procoagulant activity in the large factor VIII complex is not known. Within the human factor VIII complex, there is a high molecular weight glycoprotein,1,14-16 which supports ristocetin aggregation17,18 and forms immune precipitates in assays using heterologous antibodies to factor VIII.19,20 This complex has an associated lower molecular weight procoagulant component that is active in coagulation assays and is inhibited by human antibodies to factor VIII, though it does not form identifiable immune precipitates with these antibodies.5 The high molecular weight and low molecular weight components can be separated by gel filtration or sucrose density gradient centrifugation in the presence of high ionic strength buffers.3-5,21,22

Rabbit plasma studied by similar techniques, using physiologic ionic strength buffers, shows two peaks of factor VIII procoagulant activity, a higher molecular weight and a lower molecular weight activity (Figs. 1–3). That both procoagulants are factor VIII is indicated by immune inactivation of the procoagulant activity by two different antibodies to factor VIII (Table 1 and Fig. 4), and by the typical thrombin activation curves shown in Fig. 5.

The size heterogeneity of rabbit factor VIII activity persists when inhibitors of proteolysis are present during plasma separation and gel filtration (Fig. 2). Although these inhibitors do not prevent the appearance of two activity peaks, it is possible that the particular inhibitors and concentrations chosen may not have been adequate to prevent proteolysis of factor VIII by plasma enzymes. If the two activity peaks are not a result of proteolytic degradation, there must be at least two forms of factor VIII in normal rabbit plasma.

Like the low molecular weight factor VIII activity obtained from human plasma in high ionic strength buffers,23,24 the separated lower molecular weight component in rabbit plasma is susceptible to thrombin activation. This activation contrasts with the lack of thrombin activation that has been reported for the low molecular weight factor VIII procoagulant produced by thrombin action on human factor VIII.24 Although the smaller rabbit factor VIII activity has properties that verify its functional activity as factor VIII (Figs. 4 and 5), the slope of the low molecular weight factor VIII activity curve is different from...
normal rabbit or human plasma factor VIII activity or from the separated higher molecular weight rabbit factor VIII. The reason for this difference is not known; one might expect it to represent changes in the interaction of factor VIII with factor IX, calcium, and phospholipid.

How do the properties of the low molecular weight rabbit factor VIII activity compare to those of low molecular weight human factor VIII activity prepared in high ionic strength buffers? They are similar in that they (1) correct the coagulation defect in factor VIII-deficient plasmas, (2) can be inactivated by antibodies to factor VIII, (3) can be activated and inactivated by thrombin, and (4) have an estimated size much smaller than the estimated 1,000,000 daltons of the larger factor VIII complex.

Differences are observed, however, when centrifugation studies are carried out in high ionic strength buffers: the major component of the rabbit factor VIII activity appears to be of intermediate size, with slightly faster sedimentation properties than the human factor VIII activity under these conditions. This rabbit factor VIII activity also sediments more rapidly in 1.0 M NaCl than does the low molecular weight factor VIII of untreated rabbit plasma. A second major difference in the two (rabbit and human) small factor VIII materials has been in their activity slopes in procoagulant assays: that of high ionic strength dissociated human factor VIII activity is the same as that of normal human plasma factor VIII (unpublished observations); that of physiologic ionic strength separated rabbit low molecular weight activity is different from that of unfractionated rabbit (or human) plasma (Fig. 6).

The size heterogeneity of the rabbit factor VIII activity is important because it provides a system in which to examine the size-function relationship of the factor VIII procoagulant activity without manipulation of ionic strength.

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