Immunologic Studies of Antihemophilic Factor (AHF, Factor VIII). V. Immunologic Properties of AHF Subunits Produced by Salt Dissociation

By Margaret E. Rick and Leon W. Hoyer

Human antihemophilic factor (AHF, factor VIII), a large plasma protein with a molecular weight of approximately two million, is dissociated by changes in ionic strength. The immunologic properties of subunits obtained by sucrose density ultracentrifugation in 1 M NaCl and by agarose gel filtration in 0.24 M CaCl₂ have been determined using human and rabbit anti-AHF. Asymmetric dissociation of AHF has been identified with formation of two subunits in these separations: a nonfunctional high-molecular-weight (HMW) subunit similar in size to plasma AHF which is identified by immunoprecipitation and radioimmunoassay for AHF antigen, and an active low-molecular-weight (LMW) subunit which is not detected by these antigen assays. The LMW subunit retains AHF antigens, however, for it is inactivated by both human and rabbit anti-AHF. Antibody neutralization studies verify the presence of AHF antigens on the HMW subunit. These immunologic studies provide constraints which must be incorporated into models of AHF structure.

RECENT STUDIES HAVE indicated that antihemophilic factor (AHF, factor VIII) is present in plasma as a large protein with a molecular weight of approximately 2 million daltons. Activity is found with lower-molecular-weight proteins, however, if gel chromatography or sucrose density ultracentrifugation is carried out using high-ionic-strength buffers. The role, if any, of these subunits in normal coagulation is not established. As an initial step toward the understanding of this phenomenon, immunologic properties of AHF dissociated by high salt concentrations are reported in this paper.

Two kinds of anti-AHF have been used in these studies. Human antibodies to this procoagulant develop in some transfused hemophiliacs and may also be recognized as “spontaneous acquired anticoagulants” in rare individuals who have had no recognized prior coagulation abnormality. These antibodies can detect nonfunctional protein which has AHF antigens by antibody neutralization assays, but they do not form immune precipitates with AHF. Anti-AHF obtained from rabbits immunized with partially purified human AHF also inactivate AHF. In contrast to the human antibodies, however, they can be detected by immunoprecipitation, hemagglutination inhibition, and radioimmunoassay as well as by antibody neutralization assays.

Subunits produced by exposure of AHF to high-ionic-strength buffers have

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been characterized using both rabbit and human antibodies. Common antigenic determinants have been identified for native AHF, a nonfunctional high-molecular-weight (HMW) subunit, and an active low-molecular-weight (LMW) subunit using procoagulant inactivation and antibody neutralization assays. The native AHF and the inactive HMW subunit can also be identified by immunoprecipitation; in contrast, LMW subunits are not detected when immunoprecipitin methods are used.

**MATERIALS AND METHODS**

**Citrated Plasma**

Venous blood samples drawn from antecubital veins, using No. 18 gauge disposable needles and disposable polypropylene syringes, were added to siliconized polycarbonate tubes which contained one-ninth volume 3.8% sodium citrate. Plasma for sucrose gradient studies was separated by centrifugation at 2500g for 20 min at 4°C and was used within 60 min.

**Preparation of Purified AHF**

Ten milliliters of an AHF concentrate which contained 230 U AHF activity (Hemophil, Method Four, Hyland Laboratories, Los Angeles, Calif.) were further purified by gel filtration using Sepharose 4B (Pharmacia, Piscataway, N.J.) according to the method of Van Mourik and Mochtar. Concentrated by precipitation with an equal volume of 20% polyethylene glycol, and dissolved in 4 ml of imidazole buffered saline.

**AHF Activity Measurement**

A one-stage assay using AHF-deficient human plasma substrate (Dade, Miami, Fla.) was used. One unit of AHF is the amount present in 1 ml of average normal plasma. Pooled normal plasma served as the standard for measurement of both AHF activity and AHF antigen. It was prepared from equal volumes of freshly separated plasmas from ten normal donors. Blood for these pools was drawn into silicone-coated syringes and centrifuged at 1200 g for 15 min at 4°C in silicone-coated polycarbonate tubes. The plasmas were then recentrifuged for 15 min at 16,000 g at 4°C, pooled, and divided into small aliquots for storage at −70°C for periods of no longer than 2 mo.

AHF inactivation by human and rabbit anti-AHF was determined by incubating an equal volume of an antibody dilution with test material for 1 hr at 37°C. The residual AHF activity was then compared to that of a control sample in which barbital-saline buffer was added instead of the antibody.

**AHF Antigen Measurement**

1. Except where noted, AHF antigen measurements were determined by radioimmunoassay using 125I-labeled rabbit IgG anti-AHF. In this assay radiolabeled rabbit antibody is incubated with the test sample and the mixture is brought to a 25%, saturation with ammonium sulfate. The supernatant containing free antibody is discarded, and the precipitate (insoluble AHF-antibody complexes) is counted. The amount of radiolabeled antibody incorporated in the precipitate is proportional to the AHF antigen content of the test sample.

2. AHF antigen was also measured by the method of Zimmerman, Ratnoff, and Powell with minor modifications. Laurell electroimmunoassay was performed using 8.1 x 10 cm glass plates covered with 10 ml of 1% agarose containing 0.08 ml rabbit anti-AHF. A discontinuous buffer system was employed with barbital, μ = 0.1; pH 8.6, in the electrode vessels, and with barbital, μ = 0.05, pH 8.6, used for the preparation of the 1% agarose gel. Three-millimeter wells cut in the agarose were filled with test material and a constant voltage of 150 V was applied for 6 hr using a water-cooled system. A discontinuous buffer system was employed with barbital, μ = 0.1; pH 8.6, in the electrode vessels, and with barbital, μ = 0.05, pH 8.6, used for the preparation of the 1% agarose gel. Three-millimeter wells cut in the agarose were filled with test material and a constant voltage of 150 V was applied for 6 hr using a water-cooled system. After the plates were washed in 0.14 M NaCl and in deionized water, they were dried at 37°C and the precipitates stained with Amido-Schwartz 10B.

3. Antibody neutralization by AHF antigen was determined using human and rabbit anti-AHF according to the method of Hoyer and Breckenridge. Antibody and test material are
incubated together in this assay: a known quantity of AHF is added to the mixture, and residual AHF activity is determined after an additional 1 hr incubation at 37°C. The amount of residual AHF activity is directly related to the amount of anti-AHF neutralized by AHF antigen in the test sample. The properties of the human and rabbit antibodies used in this assay have been previously described.10,16

Sucrose Density Ultracentrifugation
Two milliliters of fresh plasma were layered on a 35-ml continuous sucrose gradient (10%, 40%, w/v) prepared with imidazole-buffered saline, and the tubes were centrifuged at 95,000 g for 22 hr at 4°C. Twelve fractions were collected by gravity from each gradient. AHF activity and AHF antigen in these fractions were compared to that of plasma incubated with 9 volumes of the gradient buffer (25%, sucrose) for 22 hr at 4°C. All assays for AHF activity were done on samples diluted 1:6 to eliminate the effects of sucrose concentration. Fractions from gradients prepared with 0.14 M NaCl were diluted with barbital-saline, and fractions from gradients prepared with 1 M NaCl were diluted with deionized water.

Agarose Gel Filtration
Samples were added simultaneously to two 1.6 x 33 cm columns of Sepharose 6B at room temperature. One was equilibrated with 0.24 M CaCl₂, 0.05 M imidazole, pH 7.2; the other with 0.12 M NaCl, 0.05 M imidazole, pH 7.2. Upward flow elution was maintained at 20 ml/hr using a two-channel peristaltic pump and 2-ml fractions were collected. AHF activity measurements for fractions from the CaCl₂ column were done after 1:12 dilution, so that the calcium ion concentration in the assay tubes was the same as that employed for all other assays.

Protein Measurements
The Lowry method was used to determine protein concentration in fractions from the sucrose density gradients.17 Protein concentration in column fractions was estimated by measurement of the optical density at 280 nm. Semiquantitative measurements of IgM, fibrinogen, and albumin were done by radial immunodiffusion.

Buffers
The barbital-saline buffer was prepared by adding 7.3 g NaCl, 2.76 g barbital, and 2.06 g Na barbital to sufficient deionized water to make 1 liter; the final pH was adjusted to 7.5 at 25°C. The imidazole buffers used for preparation of sucrose density gradients contained 0.02 M imidazole with the pH adjusted to 7.2 at 4°C. Sufficient solid NaCl was added to make the NaCl concentration 0.14 or 1.0 M. The buffers used for gel filtration studies were 0.05 M imidazole, pH 7.4 at 25°C, with additional 0.12 M NaCl or 0.24 M CaCl₂.

RESULTS
AHF activity and AHF antigen were both identified in fast sedimenting fractions when normal human plasma was fractionated by ultracentrifugation in 0.14 M NaCl. The upper panel of Fig. 1 gives data from one of four such experiments. Comparison of AHF activity values with concentrations of other plasma proteins indicates a molecular weight greater than IgM. AHF activity recovery was 44%, and AHF antigen recovery was 55% in this experiment.

Simultaneous centrifugation of plasma in 1.0 M NaCl gave a very different pattern. The lower panel of Fig. 1 gives data from one of four similar experiments. AHF activity shifted to the slowly sedimenting fractions with the activity peak between those of fibrinogen and albumin. AHF antigen remained in the fast-sedimenting fractions. AHF activity recovery was 165%, and AHF antigen recovery was 52% in this experiment; comparable values were recorded in the other three experiments.
Fig. 1. Sucrose density gradient ultracentrifugation of normal plasma. Gradients were prepared with 0.14 $M$ NaCl (upper panel) and 1.0 $M$ NaCl (lower panel) in addition to 0.02 $M$ imidazole, pH 7.2. AHF antigen was measured by radioimmunoassay.

Similar patterns of AHF activity and AHF antigen in gradient fractions were obtained when cryoprecipitates prepared from normal human plasma were centrifuged in 0.14 $M$ and 1 $M$ NaCl buffers.

AHF activity and AHF antigen are eluted in void volume fractions when plasma or AHF concentrates are chromatographed using Sepharose 6B,13 Gel filtration of highly purified AHF demonstrated this pattern if the column was equilibrated with 0.12 $M$ NaCl (Fig. 2, upper panel), but there was a separation of AHF activity from AHF antigen when the column was equilibrated with 0.24 $M$ CaCl$_2$ (Fig. 2, lower panel). Both solutions were maintained at pH 7.4 with 0.05 $M$ imidazole. The separation of AHF activity and AHF antigen by gel chromatography using 0.24 $M$ CaCl$_2$ was recognized in four successive experiments including that given in Fig. 2. There was some variation from experiment to experiment in the width of the AHF activity peak, but in every instance there was clear separation from the void volume fractions. Recovery of AHF antigen was quantitative in these experiments (e.g., in Fig. 2: upper panel, 109%; lower panel, 93%). Recovery of AHF activity was lower and was less consistent (e.g., Fig. 2: upper panel, 31%; lower panel, 35%).
As the radioimmunoassay for AHF antigen used in these experiments is based upon the insolubility of AHF antigen in 25% saturated ammonium sulfate, other methods for measurement of AHF antigen were also used. Entirely consistent data were obtained when peak fractions were assayed by Laurell electroimmunoassay. This assay is based on the formation of immunoprecipitates between antibody incorporated in the supporting medium and antigen migrating in an electric field. No immunoprecipitates were detected for low-molecular-weight fractions which had 62% of the AHF procoagulant activity of normal plasma (Fig. 3). Nonfunctional high-molecular-weight material showed typical "rocket" formation in this assay, with the length of the precipitate proportional to AHF antigen values obtained by radioimmunoassay.

AHF antigen was also identified in nonfunctional high-molecular-weight fractions by antibody neutralization studies. Void volume fractions (Fig. 2, lower panel) were tested with both rabbit and human anti-AHF (Table 1). Antibody neutralization was detected in both instances; i.e., more AHF activity was retained than with the saline controls. While quantification of antigen is much
Fig. 3. AHF antigen detected by Laurell electroimmunoassay. The fractions used were those of a gel filtration separation like that of Fig. 2. Fractions from the HMW peaks were diluted for use in this assay; the LMW fraction was not diluted. Undiluted and a 1:4 dilution of normal plasma were included for comparison.

<table>
<thead>
<tr>
<th>AHF PROCOAGULANT ACTIVITY (U/100 ml)</th>
<th>73</th>
<th>&lt;1</th>
<th>62</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHF ANTIGEN BY RADIOIMMUNOASSAY (U/100 ml)</td>
<td>112</td>
<td>122</td>
<td>&lt;5</td>
<td>100</td>
</tr>
</tbody>
</table>

less precise using this method, it is apparent that the high-molecular-weight fraction (devoid of AHF activity) has as much antibody neutralizing capacity as do hemophilic plasmas. One of the hemophilic plasmas, V.R., is CRM-positive and neutralizes both human and rabbit anti-AHF. The other, W.T., is CRM-negative and neutralizes only rabbit anti-AHF.10,13

Additional antibody neutralization studies were done using LMW subunit AHF in place of normal plasma AHF. Again, LMW and HMW fractions had common AHF antigenic determinants (Table 2).

Although the low-molecular-weight AHF procoagulant activity separated by ultracentrifugation in 1 M NaCl or gel filtration in 0.24 M CaCl₂ did not have AHF antigens detectable by radioimmunoassay (Figs. 1 and 2) or electroimmunoassay (Fig. 3), it was inactivated by both human and rabbit anti-AHF.

<table>
<thead>
<tr>
<th>Test Material</th>
<th>AHF Activity (IU/100 ml)</th>
<th>AHF Antigen (IU/100 ml)</th>
<th>%AHF Activity Retained</th>
<th>Clotting Time (sec)</th>
<th>%AHF Activity Retained</th>
<th>Clotting Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma</td>
<td>100</td>
<td>100</td>
<td>78</td>
<td>14</td>
<td>73</td>
<td>23</td>
</tr>
<tr>
<td>HMW</td>
<td>&lt;1</td>
<td>662</td>
<td>78</td>
<td>88</td>
<td>85</td>
<td>47</td>
</tr>
<tr>
<td>HMW diluted 10</td>
<td>&lt;0.1</td>
<td>66</td>
<td>88</td>
<td>30</td>
<td>93</td>
<td>16</td>
</tr>
<tr>
<td>Hemophilic plasma-W T</td>
<td>167</td>
<td>93</td>
<td>16</td>
<td>100</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>V.R.</td>
<td>35</td>
<td>186</td>
<td>89</td>
<td>22</td>
<td>93</td>
<td>14</td>
</tr>
<tr>
<td>Buffered saline</td>
<td>0</td>
<td>0</td>
<td>102</td>
<td>5</td>
<td>100</td>
<td>6</td>
</tr>
</tbody>
</table>

*For details of method see Ref 15. Test material (0.5 ml) and a dilution of antibody (0.05 ml) were incubated at 37°C for 30 min. 0.1 ml normal plasma was added as AHF source and AHF assay was done after 1 hr additional incubation at 37°C. The per cent AHF activity retained is calculated from residual AHF activity (units present in 0.65 ml volume) divided by total units AHF added in both stage I (0.5 ml test material) and stage II (0.1 ml normal plasma).

† By radioimmunoassay.

‡ See Fig. 2, lower panel. The HMW void volume fractions with maximal AHF antigen (8 ml) were pooled and dialyzed against barbital-saline prior to this assay.

§ Patient W T is CRM-negative § Patient V R is CRM-positive.
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Table 2. Cross-reactivity of LMW and HMW: Antibody Neutralization by High-Molecular-Weight AHF Subunits

<table>
<thead>
<tr>
<th>Test Material</th>
<th>AHF Activity (U/100 ml)</th>
<th>AHF Antigen (U/100 ml)</th>
<th>Clotting Time (sec)</th>
<th>%AHF Retained</th>
<th>Clotting Time (sec)</th>
<th>Human Anti-AHF %AHF Retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMW</td>
<td>&lt; 1</td>
<td>662</td>
<td>96</td>
<td>78</td>
<td>91</td>
<td>72</td>
</tr>
<tr>
<td>HMW diluted 1:10</td>
<td>&lt; 1</td>
<td>66</td>
<td>104</td>
<td>18</td>
<td>94</td>
<td>55</td>
</tr>
<tr>
<td>Buffered saline</td>
<td>0</td>
<td>0</td>
<td>112</td>
<td>5</td>
<td>101</td>
<td>20</td>
</tr>
</tbody>
</table>

*The antibody neutralization assay conditions were identical to those of Table 1 except that 0.1 ml of LMW subunits was added in place of 0.1 ml of normal plasma as AHF source in the second stage. The LMW subunits were prepared by gel filtration using 0.24 M CaCl₂ as in Fig. 2, lower panel. Fractions with maximal activity were pooled and dialyzed against barbital-saline buffer (AHF procoagulant activity was 25 U/100 ml). Normal plasma control is not included because it differs from LMW subunits in sensitivity to inactivation by rabbit and human anti-AHF (Fig. 4).

† By radioimmunoassay.

‡ See Fig. 2, lower panel. The HMW void volume fractions with maximal AHF antigen (8 ml) were pooled and dialyzed against barbital-saline prior to use in this assay.

Distinct differences were noted, however, in the inactivation of the LMW activity and plasma AHF when they were tested with dilutions of rabbit and human anti-AHF (Fig. 4). Compared with normal plasma, the LMW AHF activity was relatively resistant to the rabbit anti-AHF and more sensitive to the human anti-AHF.

DISCUSSION

Our findings are consistent with previous studies which have demonstrated a shift of AHF activity from high-molecular-weight (HMW, molecular weight greater than 1 million) fractions on sucrose density ultracentrifugation and agarose gel filtration to fractions which contain smaller proteins (LMW, molecular weights less than 200,000) when salt concentrations are changed. They suggest, as do the data of Weiss and of Owens and Wagner, that the AHF molecule consists of multiple subunits which are associated by relatively

Table 3. Effect of Antibody on AHF Activity

<table>
<thead>
<tr>
<th>AHF Source</th>
<th>Buffer</th>
<th>Clotting Time (sec) in AHF Assay†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal human plasma (1:18 dilution in 0.14 M NaCl)</td>
<td>91</td>
<td>121</td>
</tr>
<tr>
<td>Fraction 51 (0.14 M NaCl)</td>
<td>92</td>
<td>119</td>
</tr>
<tr>
<td>Normal human plasma (1:7 in 1 M NaCl)</td>
<td>87</td>
<td>109</td>
</tr>
<tr>
<td>Fraction 101 (1.0 M NaCl)</td>
<td>85</td>
<td>109</td>
</tr>
</tbody>
</table>

†Equal volumes of AHF source and antibody (or buffer) were incubated for 1 hr at 37°C. AHF assays were then performed without further dilution for 0.14 M NaCl samples. Samples in 1.0 M NaCl were diluted 1:4 with distilled water prior to assay (to adjust final NaCl concentration to 0.14 M).
weak interactions. The molecular structure is more complex than was previously recognized, however, for the identification of nonfunctional but immunologically detectable HMW protein indicates asymmetric dissociation of AHF; active LMW subunits and nonfunctional HMW subunits. These immunologic studies also establish that both subunits retain some antigenic determinants detected by rabbit and human anti-AHF.

The immunologic properties of the LMW AHF activity are different in several ways from those of plasma AHF. When compared to plasma AHF, the LMW activity is inactivated more readily by human anti-AHF, while the sensitivity to rabbit antibody is reduced. The studies reported here do not establish whether these changes represent differences in the conformation of the antigenic determinants, the effect of subunit size with parallel reduction in the number of antigenic sites per molecule, the possible presence of several different AHF specificities in the rabbit antiserum, or a combination of these effects.

While the LMW subunit retains AHF antigenic determinants, it is not detected by immunoprecipitation or radioimmunoassay. The failure of the radioimmunoassay to detect AHF antigen in these fractions is not unexpected, for LMW AHF activity is not precipitated by 25%, saturated ammonium sulfate: bound and free antibody cannot be separated when this is the case. Additional studies are necessary, however, to determine why electroimmunoassay using rabbit anti-AHF does not detect AHF antigen in the LMW fractions. It is possible that the specific activity of the LMW protein is so much greater than that of native AHF that, when samples with similar AHF procoagulant activity are
compared, there is insufficient protein in the LMW fraction to be identified by these assays. Alternatively, the LMW subunit may have only one antigenic site with which the rabbit antibody can interact. If this is the case it cannot participate in lattice formation, and no immunoprecipitate will form.

AHF antigen determinants are identified in HMW fractions, however, even though they lack AHF activity. These nonfunctional molecules have been identified by methods based on immunoprecipitation, antibody binding to macromolecular complexes (radioimmunoassay), and by antibody neutralization assays.

Taken together, these studies have demonstrated common antigenic determinants on HMW subunits and normal plasma (Table 1), HMW subunits and LMW subunits (Table 2), and LMW subunits and normal plasma (Table 3).

There is general agreement that human antibodies to AHF are functionally monospecific. Although rabbit anti-AHF have only recently been utilized for studies of this procoagulant protein, considerable data have been presented which indicates that they also react with a single plasma protein. Several laboratories have established that these antisera bind AHF, specifically neutralize AHF procoagulant activity, and form specific immune precipitates with AHF. The functionally monospecific absorbed rabbit antisera may include antibodies which detect several different AHF antigens, however. It is possible that the antibodies which react with antigens near the AHF active center, thereby inactivating LMW or plasma AHF activity, may be distinct from antibodies which bind different antigenic determinants in a way which precipitates native AHF or its HMW subunits. Absorption experiments (antibody neutralization studies—Tables 1 and 2) indicate that both subunits have antigenic determinants closely related to AHF function: it is likely that plasma AHF and HMW subunits have additional “precipitation-related” antigens as well. As noted previously, immunoprecipitation may detect these large proteins, and not the smaller LMW material, simply because they are multivalent. Further studies are needed to resolve this question.

Previous studies of AHF dissociation in high-salt buffers were discussed by Owens and Wagner with reference to two alternative models for AHF structure:

\[(AHF)_n = n(AHF) \quad (1)\]
\[Carrier (AHF)_n = Carrier + n(AHF) \quad (2)\]

Their data for canine AHF fit (2) better, for the dissociated LMW subunits did not reaggregate when they were dialyzed against normal-ionic-strength buffers. LMW subunits reassociated with the HMW material, however, when they were dialyzed together against normal-ionic-strength buffers.

Our data establish that there is asymmetrical dissociation of AHF by high salt concentrations. The demonstration of a common antigen in plasma AHF, the nonfunctional HMW subunit (by antibody neutralization), and the active LMW subunit adds an additional constraint for any AHF model. The most consistent general formulation is the following:

\[Carrier (AHF)_n = Carrier (AHF)_{n-x} + x(AHF) \quad (3)\]
Although (3) incorporates the immunoassay and antibody neutralization data, it is not entirely satisfactory, as carrier \((AHF)^{n-x}\) is not active in AHF procoagulant assays. Conformational changes may be responsible for this deficiency but there is as yet no direct evidence for this hypothesis. It is recognized that the studies reported here are also consistent with polymeric structures that are made up of similar subunits:

\[
(AHF)^n = (AHF)^{n-x} + x(AHF)
\]

The formation of active LMW subunits which are not precipitated by rabbit anti-AHF is similar in many ways to the appearance of AHF activity, also devoid of immunologic reactivity in immunoprecipitin assays, in patients with von Willebrand’s disease transfused with plasma or cryoprecipitate. Bennett, Ratnoff, and Levin reported that there is a marked difference in AHF antigen and AHF activity measurements after these infusions.²¹ AHF antigen levels return to preinfusion values by 24 hr, even though AHF procoagulant activity increases during the first 24 hr and remains elevated for 40-72 hr. The procoagulant AHF activity which appears to lack AHF antigen has properties which are similar to the LMW subunits. Additional studies are needed to consider the possibility that the in vitro production of LMW AHF activity is an appropriate model for the AHF rise in von Willebrand’s disease. Even if these phenomena are not related, the studies presented here emphasize the importance of recognizing that immunologic methods that are dependent upon secondary properties of an immune interaction are affected by many factors in addition to the presence or absence of antigenic determinants.

ACKNOWLEDGMENT

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