Immunologic Evidence That the Antihemophilic Factor (Factor VIII)-like Material in Hemophilic Plasma Possesses a Nonfunctional Low Molecular Weight Subcomponent

By Man-Chiu Poon and Oscar D. Ratnoff

Antihemophilic factor (AHF, factor VIII) is a macromolecule that can be dissociated into two subcomponents. One, of high molecular weight (MW), forms precipitates with heterologous antiserum against AHF and supports ristocetin-induced aggregation of platelets. The other, of lower MW, has procoagulant activity. The plasma of patients with classic hemophilia contains a form of AHF with properties resembling those of the high MW subcomponent. Studies of Hoyer and Breckenridge have suggested that the low MW subcomponent is present in a minority of patients whose plasmas inhibit human "circulating anticoagulant" antibodies against AHF. The present studies provide further evidence that such individuals, said to be CRM+, have a species of AHF possessing a nonfunctional variant of the low MW subcomponent. CRM+ hemophilic plasma or serum, like the low MW subcomponent of normal AHF, blocked the inhibitory properties of circulating anticoagulants, in contrast to the high MW subcomponent of normal AHF. A nonfunctional low MW subcomponent has been identified in partially purified AHF-like material separated from the plasmas of both CRM+ and CRM− hemophiliacs, the latter being patients whose plasmas do not neutralize circulating anticoagulants. This low MW subcomponent, like that of normal AHF, inhibited antiserum against the normal low MW subcomponent, but not antiserum against the normal high MW subcomponent. Thus, the defect in the hemophilic plasmas tested was not associated with the absence of the low MW subcomponent of AHF.

A variety of properties have been attributed to antihemophilic factor (AHF, factor VIII), among them the capacity to correct the coagulant defect in the plasma of patients with classic hemophilia, to support aggregation of human platelets by ristocetin, and to form immune precipitates with heterologous antisera against AHF. The plasma of patients with classic hemophilia contains a species of AHF that supports ristocetin-induced platelet aggregation and forms precipitates with specific heterologous antiserum, but is deficient in procoagulant activity. Owen and Wagner and Weiss et al. have separated normal AHF into two subcomponents. One, of high molecular weight (MW) possesses the precipitating antigen and supports ristocetin-induced platelet aggregation, while the other, which has a much lower MW, has procoagulant activity. We have presented evidence that these two...

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subcomponents are parts of the same molecule, possibly joined by noncovalent bonds.\textsuperscript{7,8}

Heterologous antisera against human AHF inactivate the procoagulant properties of AHF and its low MW subcomponent and form precipitates with AHF and its high MW subcomponent. The anticoagulant properties of such antisera can be neutralized by normal plasma and the plasma of all patients with classic hemophilia.\textsuperscript{3} In contrast, human antisera against AHF, so-called circulating anticoagulants, inactivate the procoagulant properties of AHF but do not form precipitates with this agent. The plasmas of a minority of hemophiliacs (said to have cross-reacting material, CRM+) neutralize the anticoagulant properties of these human antibodies,\textsuperscript{12,14} but in the majority of hemophiliacs (designated CRM-), plasma does not neutralize such circulating anticoagulants.

The present study suggests that the plasmas of both CRM+ and CRM- hemophiliacs have a form of AHF possessing a low MW subcomponent analogous to that of normal AHF, but lacking the capacity to sustain the normal clotting process.

\textbf{MATERIALS AND METHODS}

Citrated plasmas of normal human volunteers, three CRM- hemophilic patients, a patient with severe von Willebrand disease, and a standard pool of 24 normal plasmas were prepared and stored as previously described.\textsuperscript{3} The hemophiliacs had no detectable procoagulant AHF and had not been transfused for at least 1 mo before study. The plasma of the patient with von Willebrand disease had a procoagulant titer of 0.08 U/ml and contained 0.06 U/ml of precipitating AHF-like antigen. The pool was said to contain 1.0 U/ml each of functional (procoagulant) AHF activity and precipitating antigen. The similarity in unit measurement does not necessarily imply that all circulating AHF possesses both properties. Citrated CRM+ hemophilic plasmas from two previously reported patients\textsuperscript{12} were kindly provided by Dr. Robert T. Breckenridge, University of Rochester, Rochester, N.Y.; the titer of procoagulant AHF in these patients was 0.02 U/ml.

Partially purified AHF from normal volunteers was prepared essentially as described before.\textsuperscript{3} The crude 3\% cryoethanol precipitate prepared from 18-20 ml of plasma was redissolved in 0.8-1.0 ml of imidazole-saline buffer. One ml of this solution was filtered (without further precipitation with polyethylene glycol) at room temperature through a 0.9 x 51 cm plastic column containing 4\% agarose (Bio-Gel A15M, Bio-Rad Laboratories, Richmond, Calif.) that was equilibrated and eluted with imidazole-saline buffer at a constant pressure at the outflow of 30 cm of water, collecting 1.2 ml fractions. The AHF-rich fractions excluded by the gel (usually fractions 12-15) were pooled and concentrated to 0.5-0.6 ml by negative pressure dialysis at 4°C through cellophane tubing (1-in diameter, Fisher Scientific Co., Pittsburgh, Pa.) against barbital-saline buffer.

The analogous fraction of AHF-like material was prepared in an identical fashion from the plasma of three individuals with CRM- and one with CRM+ classic hemophilia, using the same agarose column. As described earlier, the AHF-like precipitating antigenic material of hemophiliacs, like that of normal individuals, was excluded by the agarose gel.\textsuperscript{15} The hemophilic fractions containing AHF-like material were pooled and concentrated. The plasma of a patient with von Willebrand disease was treated in a similar fashion. In other experiments, partially purified AHF from normal volunteers or CRM- hemophiliacs was prepared from plasma derived from 250 ml of citrated blood by filtering the crude fraction of plasma insoluble in 3\% ethanol and 10\% polyethylene glycol (MW 6000, Fisher Scientific Co., Fair Lawn, N.J.) through a 2.5 x 40 cm 4\% agarose column.\textsuperscript{3}

Separation of the high and low MW subcomponents of partially purified AHF derived from individual normal donors was conducted essentially by the method of Owen and Wagner.\textsuperscript{5} A concentrated preparation of partially purified AHF (0.5 ml) was applied to a 0.9 x 26 cm plastic column containing 8\% agarose (Bio-Gel A1.5M, 200-400 mesh, Bio-Rad Laboratories) that was
equilibrated and eluted with Tris-calcium chloride buffer at a constant pressure at the outflow of 30 cm of water; 0.85-ml fractions were collected at room temperature. The fractions were immediately assayed for procoagulant activity. The high MW subcomponent, containing precipitating antigens, was present in fractions excluded by the agarose gel, usually fractions 9-11, whereas the low MW subcomponent (presumed MW of about 230,000) containing AHF procoagulant activity was eluted in later fractions, usually fractions 14-17. The two subcomponents were pooled separately and concentrated to 0.5-0.6 ml by negative pressure dialysis in 1/4-in diameter cellophane tubing against barbital-saline buffer for determination of protein and antigen and for absorption of heterologous and human antisera against AHF.

Concentrates of partially purified AHF-like material prepared from hemophilic or von Willebrand disease plasmas were gel filtered through the same 8% agarose column, equilibrated with Tris-calcium chloride buffer. Fractions at the same position at which normal high and low MW subcomponents were eluted were similarly pooled and concentrated for determination of protein and precipitating antigen and absorption of heterologous and human antisera against AHF.

Partially purified AHF from commercially available AHF concentrates of plasma, Factorate (Armour Pharmaceutical Co., Kankakee, Ill.), Hemofil (Hyland Corp., Costa Mesa, Calif.), and Profilate (some the gift of Abbott Laboratories, North Chicago, Ill.), were prepared and separated into high and low MW subcomponents as previously described. The high MW subcomponent separated in this way was usually further purified by a second filtration through an 8% agarose column in the presence of Tris-calcium chloride buffer, a procedure necessary to reduce its content of agents with properties associated with the low MW subcomponent.

Procoagulant AHF was measured by an earlier technique. The samples eluted in Tris-calcium chloride buffer from the 8% agarose column were assayed by modification of an earlier method, omitting the addition of protamine sulfate. A mixture of 0.1 ml of a substrate of hemophilic plasma and 0.1 ml of 0.1% Centrolex “O” or “R” (crude soybean phosphatides, the gift of Central Soya Chemurgy, Chicago, Ill.) in 0.15 M sodium chloride was incubated at 37°C for 8 min. Thereafter, 0.1 ml of barbital-saline buffer, prewarmed to 37°C, and 0.1 ml of the sample to be tested, diluted 1/10 in barbital-saline buffer (and thus containing 0.025 M calcium chloride), were added in rapid succession, and the clotting time was measured.

Rabbit antisera to concentrates of partially purified normal AHF, and to its high and low MW subcomponents, and goat antiserum against normal AHF were prepared as previously described. The properties of the rabbit antisera toward various normal plasma AHF fractions have been described in detail. Thus, rabbit antiserum to normal AHF and to the normal low MW subcomponent inactivated procoagulant AHF in normal plasma, partially purified preparations of normal AHF, and the normal low MW subcomponent. The clot-inhibitory activities of these antisera (i.e., their capacity to inactivate the procoagulant properties of AHF) were blocked by antigens in all these preparations and by the normal high MW subcomponent as well (Table 2A). In contrast, rabbit antiserum against the normal high MW subcomponent inactivated procoagulant AHF in normal plasma and partially purified preparations of normal AHF, but was without effect upon the normal low MW subcomponent. The clot-inhibitory activity of this antiserum against the high MW subcomponent was blocked by partially purified preparations of normal AHF and the normal high MW subcomponent, but not by the normal low MW subcomponent (Table 2A).

Rabbit antisera against concentrates of partially purified AHF-like material in CRM- hemophilic plasma were prepared and absorbed as described by Bennett et al. These antisera formed a line of identity upon immunodiffusion against normal and hemophilic plasmas.

Crude immunoglobulin fractions were prepared from the plasma of five patients with circulating anticoagulants and from various heterologous antisera, using a minor modification of the method of Steinbuch and Audran. Two of the patients whose plasmas contained circulating anticoagulants were hemophilic, while in the other three this abnormality appeared to be “acquired.”

The effect of rabbit antisera upon the procoagulant properties of AHF in plasma, partially purified normal AHF, and its low MW subcomponent was examined as in earlier studies. Blocking of the clot-inhibitory properties of rabbit antisera by preparations of normal AHF and the AHF-like fraction of CRM− or CRM+ hemophilic plasma or von Willebrand disease plasma, and by the high and low MW subcomponents separated from these preparations, was tested by a minor modification of an earlier method. A volume of 0.025 ml of rabbit antiserum, or non-immune normal rabbit serum, suitably diluted in barbital-saline-albumin buffer, was incubated with 0.075 ml of the agents to be tested in 10 x 75 mm polystyrene tubes for 1 hr at 37°C and
then at 4°C overnight. Each mixture was then incubated at 37°C for 1 hr to inactivate any residual functional AHF and centrifuged at 1300 g (as measured at the tube bottom) at 2°C for 15 min. A 0.075-ml sample of the supernatant mixture was incubated in 10 × 75 mm polystyrene tubes for 1 hr at 37°C with 0.075 ml of pooled normal plasma and diluted 1/10 in barbital-saline buffer; residual procoagulant AHF was then measured by the method described.

Neutralization of the inhibitory activity of human circulating anticoagulants against AHF was demonstrated by a modification of the method of Hoyer and Breckenridge.10 0.1 ml of the agent to be tested was incubated at 37°C for 1 hr in 10 × 75 mm borosilicate glass tubes with 0.05 ml of a crude immunoglobulin fraction of a plasma with circulating anticoagulant activity, suitably diluted in barbital-saline-albumin buffer. Thereafter, 0.05 ml of normal pooled plasma (indicator plasma) was added to the incubation mixture and incubated at 37°C for 1 hr, and residual functional AHF activity of the indicator plasma was then measured. The concentration of the immunoglobulin fraction used was such that (1) its anticoagulant activity was incompletely neutralized by normal pooled plasma, and (2) the residual anticoagulant after incomplete neutralization would not inactivate the indicator plasma completely when CRM− hemophilic plasma or buffer was incubated with the immunoglobulin fraction.

When the agents to be tested were plasma or serum, the controls consisted of normal pooled plasma, CRM− plasma, and different dilutions of normal pooled plasma in the CRM− plasma. Thus, the neutralizing activity of the plasma or serum under study could be compared with that of various concentrations of normal plasma in CRM− hemophilic plasma. When partially purified plasma fractions diluted in buffer were tested, an additional control of buffer was also used. When a potent circulating anticoagulant was used, a linear relationship was usually obtained when the logarithm of residual functional AHF activity in the indicator plasma was plotted against the concentration in the initial mixture of normal pooled plasma diluted with CRM− plasma. When weak anticoagulants were tested, the standard curve was too shallow for accurate quantification.

Recalcified serum for neutralization of circulating anticoagulants was prepared by incubating citrated plasma with 1/20 volume of 0.5 M calcium chloride at 37°C for 1 hr. After removal of the fibrin clot by centrifugation at 2500 g at 2°C for 15 min, the excess calcium chloride was removed by exhaustive dialysis against barbital-saline buffer at 4°C. A control was also prepared in which barbital-saline buffer was substituted for 0.5 M calcium chloride in the procedure.

A thrombin-inactivated normal low MW subcomponent of AHF was prepared by incubating 0.39 ml of the low MW subcomponent in barbital-saline buffer at 37°C for 1 hr with 0.01 ml of 2 NIH units of crude bovine thrombin (Topical Thrombin, Parke Davis Co., Detroit, Mich.) per milliliter of the same buffer. Thrombin at this concentration (final concentration 0.05 NIH U/ml) inactivated the functional AHF activity of the low MW subcomponent completely without visibly clotting the plasma.

Precipitating antigen related to AHF was measured by an earlier adaptation3 of Laurell's method4 of semiquantitative immunoelectrophoresis using antiserum to human AHF raised in a "grade" goat and absorbed to remove extraneous antibodies.7 The technique had a sensitivity of 0.025–0.05 units of antigen/ml plasma. Immunodiffusion was performed as previously described.3

Barbital-saline buffer was composed of 0.025 M sodium barbital and 0.125 M sodium chloride at pH 7.5. Barbital-saline-albumin buffer contained an additional 2 mg human serum albumin (Schwartz-Mann, Orangeburg, N.Y.) per milliliter. Imidazole-saline buffer was 0.02 M imidazole (Matheson, Coleman and Bell, Norwood, Ohio) in 0.14 M sodium chloride at pH 6.5. Tris-calcium chloride buffer contained 0.05 M tris-(hydroxymethyl) aminomethane (Sigma Chemical Co., St. Louis, Mo.) in 0.25 M calcium chloride at pH 7.35. All buffers for gel filtration contained 200 mg/liter sodium azide.

Protein concentration was measured by the method of Lowry et al.20

Centrifugation at room temperature was carried out in an International UV centrifuge and at 2°C in an International PR-2 centrifuge.

RESULTS

As reported previously by Hoyer and Breckenridge,12,18 in this study the inhibitory activity of each of five circulating anticoagulants against AHF was
Table 1. Neutralization of Human Circulating Anticoagulants by Plasma, Serum, and Fractions of Partially Purified Normal AHF

<table>
<thead>
<tr>
<th>Neutralizing Agent</th>
<th>Neutralization Capacity (%)</th>
<th>No. of Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Normal Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>95.8 ± 3.9</td>
<td>5</td>
</tr>
<tr>
<td>CRM+ Plasma no. 1</td>
<td>63.4 ± 6.1</td>
<td>3</td>
</tr>
<tr>
<td>Serum no. 1</td>
<td>65.7 ± 5.1</td>
<td>3</td>
</tr>
<tr>
<td>CRM+ Plasma no. 2</td>
<td>72.7 ± 9.1</td>
<td>3</td>
</tr>
<tr>
<td>Serum no. 2</td>
<td>75.7 ± 5.1</td>
<td>3</td>
</tr>
<tr>
<td>CRM- Plasma</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>vWD Plasma</td>
<td>10.0, 6.0</td>
<td>2</td>
</tr>
<tr>
<td>B. Normal High MW subcomponent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low MW subcomponent (not treated with thrombin)</td>
<td>68.0 ± 9.5</td>
<td>3</td>
</tr>
<tr>
<td>Low MW subcomponent (treated with thrombin)</td>
<td>65.0 ± 9.1</td>
<td>3</td>
</tr>
<tr>
<td>CRM+ High MW subcomponent</td>
<td>3.0, 0</td>
<td>2</td>
</tr>
<tr>
<td>Low MW subcomponent</td>
<td>34.0, 38.0</td>
<td>2</td>
</tr>
<tr>
<td>CRM- High MW subcomponent</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Low MW subcomponent</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>vWD High MW fraction</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Low MW fraction</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Values are means ± 1SD.

One-tenth ml of the neutralizing agent was incubated at 37°C for 1 hr with 0.05 ml of the circulating anticoagulant. Thereafter, 0.05 ml of normal pooled (indicator) plasma was added and incubated at 37°C for 1 hr more and the residual fraction AHF of the indicator plasma was then measured. The degree of neutralization was compared with that of different dilutions of normal pooled plasma in CRM- hemophilic plasma and was expressed as the percentage of the equivalent normal pooled plasma. The results shown here were obtained with one circulating anticoagulant, but those obtained with the other four less potent circulating anticoagulants showed similar patterns. In section A, the normal plasma contained 1 procoagulant AHF U/ml and 1 antigen U/ml. The CRM+ hemophilic plasma no. 1 contained 0.82 antigen U/ml and the CRM+ hemophilic plasma no. 2 contained 1.02 antigen U/ml. The von Willebrand disease (vWD) plasma contained 0.08 procoagulant AHF U/ml and 0.06 antigen U/ml. All sera were prepared as described in Materials and Methods.

In section B, the normal high MW subcomponent contained 3.0 antigen U/ml (68.6 antigen U/mg protein). The normal low MW subcomponent contained 0.4 procoagulant AHF U/ml (24.5 procoagulant AHF U/mg protein). The low MW subcomponent was inactivated by incubating 0.39 ml at 37°C for 1 hr with 0.01 ml 2.0 NIH U/ml bovine thrombin (final concentration 0.05 NIH U/ml). The control active low MW subcomponent was similarly treated with barbital-saline buffer instead of thrombin. The CRM+ hemophilic high MW subcomponent contained 0.47 antigen U/ml and approximately 15 µg protein/ml; the CRM+ hemophilic low MW subcomponent was devoid of antigen and contained approximately 5 µg protein/ml. The CRM- hemophilic high MW subcomponent contained 0.44 antigen U/ml and approximately 17 µg protein/ml; the CRM- hemophilic low MW subcomponent was devoid of antigen and contained approximately 5 µg protein/ml. Both the high and low MW fractions of vWD AHF were devoid of procoagulant activity and antigen; the high MW fraction contained approximately 5 µg protein/ml and the low MW subcomponent 3 µg protein/ml.

The normal high and low MW subcomponents were separated from partially purified AHF prepared from Factorate (Armour); other preparations were made from plasma.

Neutralized by normal plasma, the plasmas of two previously reported CRM+ hemophiliacs, and partially purified normal AHF. "Cross-reacting material" that neutralized each of the circulating anticoagulants was also present in diminished amounts in the sera of normal individuals and CRM+ patients with hemophilia (Table 1A), in confirmation of studies by Hoyer and Breckenridge. Similar blocking activity was demonstrated by the normal low MW subcomponent of AHF, even when its procoagulant activity had been destroyed by thrombin (Table 1B). Thus, the antigenicity of the low MW subcomponent
was not dependent upon its procoagulant properties. In contrast, twice-filtered high MW subcomponents of normal AHF, devoid of procoagulant activity, did not neutralize the activity of any of the five circulating anticoagulants. The low MW, but not the high MW subcomponent of the AHF-like material of CRM+ hemophilic plasma neutralized the circulating anticoagulants (Table 1B). Thus, the cross-reacting material in the plasma of CRM+ hemophiliacs appeared to reside in the fraction of plasma analogous to that containing the low MW subcomponent of AHF-like material, but at a site independent of its functional activity.

The experiments just described indicate that the plasma of patients with CRM+ hemophilia has properties resembling those of the low MW subcomponent of AHF. The following experiments suggest that this is also true of the plasma of CRM− patients.

Earlier we reported15 that upon immunodiffusion, an antiserum to the noncoagulant agent in CRM− hemophilic plasma analogous to normal AHF formed a line of identity between normal and hemophilic plasma, and that such antiserum inhibited the procoagulant properties of partially purified AHF. Further studies have demonstrated that such antisera also inhibit the procoagulant activity of the functional low MW subcomponent of normal AHF (Fig. 1). The antiserum tested was weaker than similar preparations of antiserum against normal AHF, but the significance of this difference is not certain.15

Consistent with these observations, the antiserum against the nonfunctional AHF-like fraction of hemophilic plasma was neutralized by absorption with partially purified normal AHF or its high or low MW subcomponents, and by the analogous fractions of CRM− hemophilic plasma (Table 2A).

Like normal AHF,7 the partially purified AHF-like material separated from each of three CRM− hemophilic plasmas blocked the clot-inhibitory effect of heterologous antisera against normal AHF, or its normal high or low MW subcomponents. A typical example of four replicate studies is shown in Table 2A. This AHF-like fraction was separated into high and low MW subcomponents. These subcomponents of hemophilic AHF-like material behaved like their normal analogues in blocking experiments: the high MW subcomponent blocked antisera against normal AHF or against its high or low MW subcom-
Table 2. Blocking Effects of Normal AHF, Hemophilic “AHF,” von Willebrand Disease “AHF,” and Their High and Low MW Subcomponents Upon Heterologous Antiserum to These Agents

<table>
<thead>
<tr>
<th>Blocking Agent</th>
<th>Residual AHF (U/ml) After Incubation With Antiserum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Barbitol-saline buffer</td>
<td></td>
</tr>
<tr>
<td>Normal AHF no. 1</td>
<td>0.15</td>
</tr>
<tr>
<td>Normal low MW subcomponent</td>
<td>0.49</td>
</tr>
<tr>
<td>Normal AHF no. 2</td>
<td>0.46</td>
</tr>
<tr>
<td>Normal high MW subcomponent</td>
<td>0.40</td>
</tr>
<tr>
<td>CRM+ hemophilic “AHF”</td>
<td>0.43</td>
</tr>
<tr>
<td>CRM- hemophilic “AHF”</td>
<td>0.40</td>
</tr>
<tr>
<td>vWD AHF</td>
<td>0.18</td>
</tr>
<tr>
<td>B. Barbitol-saline buffer</td>
<td></td>
</tr>
<tr>
<td>vWD low MW fraction</td>
<td>0.24</td>
</tr>
<tr>
<td>vWD high MW fraction</td>
<td>0.23</td>
</tr>
<tr>
<td>CRM+ low MW subcomponent</td>
<td>0.36</td>
</tr>
<tr>
<td>CRM+ high MW subcomponent</td>
<td>0.63</td>
</tr>
<tr>
<td>C. Barbitol-saline buffer</td>
<td></td>
</tr>
<tr>
<td>CRM- low MW subcomponent</td>
<td>0.09</td>
</tr>
<tr>
<td>CRM- high MW subcomponent</td>
<td>0.25</td>
</tr>
<tr>
<td>CRM- high MW subcomponent</td>
<td>0.49</td>
</tr>
</tbody>
</table>

*Antiserum was incubated with the blocking agent and heated at 56°C. Thereafter, a sample was incubated at 37°C for 1 hr with diluted pooled normal plasma, and the residual AHF activity was then measured (see Materials and Methods). Before addition of the blocking agent, antiserum against AHF was diluted 1/25, against the normal low MW subcomponent 1/8, and against CRM- hemophilic AHF 1/8, with barbital-saline-albumin buffer. In section A, partially purified normal AHF no. 1, containing 1.0 procoagulant AHF U/ml (31.7 procoagulant AHF and 40.0 antigen U/mg protein), was compared to the normal low MW subcomponent, containing 1.0 procoagulant AHF U/ml (31.2 procoagulant AHF U/mg protein). Normal AHF no. 2, containing 2.0 antigen U/ml (specific activity same as AHF no. 1), was compared (1) to the normal high MW subcomponent, containing 2.0 antigen U/ml (66.3 antigen U/mg protein), (2) to the partially purified CRM+ hemophilic AHF, containing 2.0 antigen U/ml (25.2 antigen U/mg protein), and (3) to the partially purified CRM- hemophilic AHF, containing 2.0 antigen U/ml (27.8 antigen U/mg protein). The fraction of vWD plasma analogous to normal AHF contained 0.08 procoagulant AHF U/ml, 0.14 antigen U/ml, and approximately 25 μg protein/ml.

In section C, the CRM- hemophilic low MW subcomponent was devoid of antigen and contained approximately 5 μg protein/ml, the CRM- hemophilic high MW subcomponent contained 0.42 antigen U/ml and approximately 18 μg protein/ml. The normal high and low MW subcomponents were separated from partially purified AHF prepared from Profilate (Abbott); all other preparations were made from plasma.

In contrast, the low MW subcomponents neutralized antisera to the normal low MW subcomponent, but not to the high MW fraction. A typical example among seven replicate studies is presented in Table 2C. Thus, a similarity in antigenicity was demonstrated between the low MW subcomponent of normal AHF and its CRM- hemophilic analogue. Similar data were obtained in studies of the analogous fraction of CRM+ hemophilic plasma (Table 2A,B), in agreement with the evidence presented that such plasma contains an analogue of the low MW subcomponent of normal AHF.

The partially purified fraction of von Willebrand disease plasma analogous to that of normal AHF exhibited negligible blocking activity toward the various heterologous antisera tested (Table 2A). Its high MW and low MW fractions,
devoid of detectable precipitating antigens and procoagulant activity, did not neutralize these antisera (Table 2B).

DISCUSSION

Hemophilic plasma contains AHF-like material that brings about the formation of precipitates with heterologous antiserum against AHF and supports ristocetin-induced platelet aggregation. These properties are also attributes of the high MW subcomponent of normal AHF. Several lines of evidence presented here suggest that hemophilic plasma also contains an analogue of the low MW subcomponent of normal AHF that is nonfunctional in clotting assays.

Hoyer and Breckenridge and others have demonstrated that human "circulating anticoagulant" antibodies against AHF are neutralized not only by normal plasma, containing Al-IF, but also by that of a minority of hemophiliacs, whose plasmas are therefore said to contain cross-reacting material. This cross-reacting material has been detected in undiminished amounts in sera of such patients (said to be CRM+), whether obtained from spontaneously clotted blood or by recalcification of plasma. The majority of hemophiliacs, said to be CRM−, lack cross-reacting material detected by this method. Biggs, however, has reported that she could demonstrate the presence of cross-reacting AHF-like antigens in CRM− patients; the validity of this observation is uncertain because necessary control tests were not described.

In the experiments reported herein, we demonstrated that the low MW component of normal plasma neutralized the anticoagulant properties of five circulating anticoagulants against AHF. This neutralizing property was retained in low MW subcomponents of AHF whose procoagulant properties were destroyed by incubation with small amounts of thrombin. In contrast to studies reported by Rick and Hoyer, the high MW subcomponent of normal AHF did not neutralize these circulating anticoagulants. The difference in the results obtained may be attributable to the fact that our high MW subcomponents had been filtered twice through agarose gels.

These observations suggest that the ability to neutralize human circulating anticoagulants is a valid marker for the presence of low MW-like material, whether or not functional procoagulant activity is present. These findings provide indirect evidence that the AHF-like material of CRM+ hemophilic plasmas possesses a low MW subcomponent that is analogous to that of normal AHF, but is incompetent in procoagulant functions.

Heterologous antiserum against the hemophilic analogue of AHF, prepared from the plasmas of patients lacking cross-reacting properties against circulating anticoagulants (i.e., CRM−) behaved like antisera against normal AHF or its low MW subcomponent. Unlike antiserum against the normal high MW subcomponent of AHF, it inactivated the procoagulant properties of the normal low MW subcomponents of AHF, and this antigen neutralized the clot-inhibitory properties of the antiserum.

When the partially purified analogue of AHF, prepared from three CRM− and one CRM+ hemophilic plasma, was gel filtered through 8% agarose in the presence of 0.25 M calcium chloride, a fraction excluded by the gel (like
LMW SUBCOMPONENT OF AHF

the analogous normal high MW subcomponent) possessed the bulk of protein, precipitated heterologous antiserum against AHF, and blocked the clot-inhibitory activities of antisera against normal AHF and its subcomponents. Furthermore, fractions of AHF eluted at the same position as the normal low MW subcomponent and devoid of precipitating antigens blocked clot-inhibitory properties of rabbit antiserum against normal AHF or the normal low MW subcomponent. In contrast, like the normal analogue, the hemophilic low MW subcomponent did not block rabbit antiserum against the normal high MW subcomponent. These observations imply that the low MW subcomponent separated from hemophilic plasma was analogous to that of normal AHF, and that it was not appreciably contaminated with the high MW subcomponent.

Thus our data suggest the presence of an analogue of the low MW subcomponent of AHF in the plasma of three CRM- and two CRM+ hemophiliacs studied. The nature of the defect in these hemophilic low MW subcomponents that resulted in a loss of procoagulant function could not be resolved. Our observations are consistent with the view that the antigenic site in CRM+ hemophilic plasma that reacts with circulating anticoagulants is distinct from that associated with procoagulant activity in normal plasma, as it was not affected by incubation with thrombin, which destroys the coagulant properties of AHF.

The data we have obtained did not provide information as to whether other physical or chemical properties of the abnormal low MW subcomponents in our patients were similar to those of normal AHF. For instance, the point of maximal elution from agarose columns was unknown, as the fractions selected for study were those which would have contained normal low MW subcomponents of AHF. Our experiments did not provide clues as to whether the stoichiometric relationship between the high and low MW subcomponents of hemophilic plasma is similar to that of normal AHF. Furthermore, the limited number of patients studied does not allow us to assume that the AHF-like material of all hemophilic plasmas possesses a low MW subcomponent. The existence of CRM- and CRM+ variants of hemophilia caution us to anticipate other forms of heterogeneity in hemophilic patients. The observation of Cooper and Wagner that the low MW subcomponent of normal plasma appeared to form complexes with hemophilic AHF may be interpreted to mean that the hemophilic AHF they studied was deficient in the low MW subcomponent.

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Immunologic evidence that the antihemophilic factor (factor VIII)-like material in hemophilic plasma possesses a nonfunctional low molecular weight subcomponent

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