Immunologic Studies of Antihemophilic Factor (AHF, Factor VIII). III. Comparative Binding Properties of Human and Rabbit Anti-AHF

By Leon W. Hoyer

The binding properties of human and rabbit antibodies to antihemophilic factor (AHF, factor VIII) have been compared in experiments that sought the basis for their different specificities and secondary properties. Agarose gel filtration demonstrated the formation of stable complexes of AHF with rabbit anti-AHF but not with human anti-AHF. Measurement of bound radiolabeled rabbit anti-AHF provides a very sensitive assay for the AHF antigen that is present in normal and hemophilic plasmas but that is markedly reduced in plasmas from patients with von Willebrand's disease.

Recent immunologic studies have demonstrated that several different molecular defects may cause reduced plasma antihemophilic factor (AHF, factor VIII) activity in hemophilia A (classical hemophilia). Human antibodies that inactivate AHF (obtained from transfused hemophiliacs or from patients with spontaneous anti-AHF anticoagulants) are neutralized by nonfunctional AHF-like molecules found in plasmas from approximately 10% of patients with hemophilia A.1–3 Similar studies with rabbit anti-AHF have given very different results, however, for they have demonstrated immunologically cross-reacting AHF-like molecules in all hemophilic plasmas.4,5 These rabbit antibodies, obtained from animals immunized with purified AHF preparations, may be detected by precipitation4 or by hemagglutination inhibition5 methods, as well as by the more difficult assays based on anticoagulant neutralization.

The binding studies reported in this paper have compared the two kinds of anti-AHF and have identified a basis for the different reactivities. These experiments have demonstrated that rabbit anti-AHF forms a stable complex with AHF. In contrast, human anti-AHF inactivates the coagulation factor even though it does not form a stable complex with AHF. The very different secondary properties (precipitation, hemagglutination) of the two kinds of anti-AHF are a reflection of these binding differences.

From the Department of Medical and Pediatric Specialties, University of Connecticut School of Medicine, Hartford, Conn. and the Medical Service, Veterans Administration Hospital, Newington, Conn.

Submitted July 1, 1971; August 20, 1971; October 11, 1971.

Supported by a Veterans Administration Research Grant.

Leon W. Hoyer, M.D.: Associate Professor of Medicine, University of Connecticut School of Medicine, Hartford, Conn., and Consultant in Hematology, Veterans Administration Hospital, Newington, Conn.
MATERIALS AND METHODS

Citrated Plasmas

Venous blood samples were drawn from antecubital veins using No. 18 gauge disposable needles and disposable polypropylene syringes and added to silicone-coated polycarbonate tubes that contained one-ninth volume 3.8% sodium citrate. Plasma was separated by centrifugation at 1200 g for 15 min at 4°C and then respun at 16,000 g for 15 min at 4°C. The normal plasma used in binding studies was prepared by pooling equal volumes of freshly separated plasma from ten normal subjects. Aliquots of this mixture were kept at —70°C in silicone-coated polystyrene tubes.

Preparation of AHF-Rich Concentrates

AHF-rich concentrates were prepared from individual normal plasmas by adding sufficient 80% ethanol to bring the final ethanol concentration to 3%. After gentle stirring of the plasma at —3°C for 30 min, the mixture was centrifuged at 16,000 g for 10 min at —3°C. The precipitate was dissolved in imidazole saline buffer (0.02 M imidazole, 0.14 M NaCl, pH 6.5) using one-tenth the volume of the starting plasma sample.

Preparation of Purified AHF

Highly purified AHF was obtained from fresh normal plasma by precipitation at —3°C with 3% ethanol, precipitation with 10% polyethylene glycol, and gel filtration through Sepharose 4B (Pharmacia, Piscataway, N.J.) following the method of Zimmerman et al.4 The AHF-rich fractions that were eluted at the void volume were concentrated by ultrafiltration and mixed with an equal volume of aluminum hydroxide gel [a 1:10 dilution in saline of Rehsorptar (Armour Pharmaceutical Co., Chicago, Ill.)] New Zealand albino rabbits were immunized with 0.5 ml of this mixture (1–2 U AHF and 10-25 μg protein/ml) in each hind footpad. Blood obtained by ear artery puncture was allowed to clot at 37°C, and the serum was separated. The antiserum used in the studies reported here was obtained after a total of four injections over a 7-mo period. The serum was heated to 56°C for 30 min, clarified by centrifugation at 2,000 g for 15 min at 4°C, and absorbed with 10 mg calcium triphosphate (J. T. Baker, Philipsburg, N.J.) per ml of plasma for 10 min at 25°C before centrifugation at 2,000 g for 15 min at 4°C.

This serum contained antibodies to at least eight human plasma proteins when tested by immunoelectrophoresis. Absorption of the rabbit antiserum with an AHF-poor fraction of human plasma removed all of the precipitating antibodies except those that reacted with a single plasma component. The AHF-poor absorbing antigen was prepared by bringing the supernatant plasma from the 3% ethanol —3°C separation to a final ethanol concentration of 8% at —3°C. To the precipitate, separated by centrifugation at 16,000 g for 10 min at —3°C, was added the heated and calcium phosphate-absorbed rabbit anti-serum equal in volume to one-half the 3% ethanol supernatant used to obtain the absorbing material. The mixture was incubated at 37°C for 1 hr and at 4°C overnight before centrifugation at 16,000 g for 10 min at —3°C. The absorbed serum was then tested by Ouchterlony gel diffusion and by immunoelectrophoresis against normal plasma and AHF-rich concentrates of normal plasma. A single line was demonstrated in both instances. The absorbed antiserum had the same capacity to neutralize AHF in normal plasma as did the rabbit serum from which it was prepared.

IgG was isolated from the serum of a patient (A. R.) who developed an anti-AHF after a penicillin reaction,6,7 from the absorbed rabbit anti-AHF, from normal human serum, and from normal rabbit serum. Whole globulin fractions were obtained from sera by three precipitations with ammonium sulfate. Proteins that precipitated in 50% saturated ammonium sulfate at 25°C, pH 7.9, were dissolved in saline and dialyzed against 0.04 M phosphate, pH 8.9. IgG was separated from these whole globulin preparations by column chromatography using DEAE-cellulose (DE-52, Reeve-Angel, Clifton, N.J.) equilibrated with the same buffer. The IgG prepared from human globulins had a single line on immunoelectrophoresis when tested with a rabbit anti-whole human serum. The flow-through
peak of the rabbit globulin fractions was essentially pure IgG (with trace contamination by transferrin) when tested with sheep anti-whole rabbit serum.

The purified IgG fractions were labeled with $^{125}$I or $^{131}$I (New England Nuclear, Boston, Mass.) by the iodine monochloride (ICl) method. The labeled proteins were dialyzed against barbital-saline buffer and stored at $-20^\circ$C until used. Over 98% of the radioactivity was precipitable by 10% trichloroacetic acid (TCA), and the labeled proteins had 0.2–0.8 iodine atoms per molecule with specific activities of 35–205 $\mu$Ci/mg.

Antihemophilic Factor Assay

Antihemophilic factor was assayed by a one-stage method using AHF-deficient human plasma substrate (Dade, Miami, Fla.). The pooled normal plasma, stored at $-70^\circ$C for periods up to 2 mo, was the standard used in these studies. All clotting studies were performed in uncoated glass tubes with an internal diameter of 8 mm. All AHF values are expressed in the units proposed by an ad hoc committee of the National Research Council in which 1 U of AHF is the amount in 1 ml of average normal plasma.

Anti-AHF Neutralizing Activity

Anti-AHF neutralizing activity was measured by incubating the test material with an equal volume of normal human plasma for 2 hr at $37^\circ$C. The AHF activity of this mixture was then determined, and the percentage neutralization calculated by comparing residual AHF activity with that of a control mixture of normal human plasma incubated with barbital-saline buffer.

Binding Studies

Binding studies were done at $25^\circ$C using a 1.6 $\times$ 60 cm column of Sepharose 6B equilibrated with barbital-saline buffer. The mixtures of plasma or AHF-rich concentrate with radiolabeled IgG were incubated at $37^\circ$C for 30 min before the gel filtration. The addition of 0.25 ml normal rabbit plasma to the incubation mixture did not affect the elution properties of either rabbit or human radiolabeled IgG. It was, therefore, included in all mixtures that had less than 0.1 ml of human plasma or concentrate to avoid any artifacts associated with low protein concentration. Upward-flow elution was maintained at 20 ml/hr using a peristaltic pump. Two-milliliter fractions were collected in polystyrene tubes that were counted using a two-channel crystal scintillation detector (Nuclear-Chicago Corp., Des Plaines, Ill.). The protein content of these fractions was estimated from the absorption at 280 nm. The void volume of this column (40 ml) was established using Blue Dextran (Pharmacia).

Immunodiffusion was done using microslides coated with 1% agar. The barbital-saline buffer, pH 7.4–7.5, contained 7.3 g NaCl, 2.76 g barbital, 2.06 g sodium barbital, and distilled water to make the volume 1 liter.

RESULTS

The rabbit anti-AHF was obtained from animals repeatedly immunized with purified AHF. This antiserum contained antibodies against several plasma proteins and was, therefore, absorbed with an AHF-depleted ethanol fraction of normal human plasma. The absorbed antiserum formed a single line in gel diffusion analysis when tested with normal human plasma or with AHF-rich concentrates. Zimmerman et al. have reported the properties of antibodies prepared in this manner, and they concluded from their data that the gel diffusion precipitates identify AHF. As expected, the rabbit antibody had no effect on rabbit AHF activity.

The human anti-AHF was obtained from the serum of a patient who developed a “spontaneous” antibody to AHF after a penicillin reaction. The proper-
Table 1. AHF Neutralization by Radiolabeled IgG Anti-AHF

<table>
<thead>
<tr>
<th></th>
<th>Residual AHF* (U/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-IgG Rabbit anti-AHF</td>
<td></td>
</tr>
<tr>
<td>0.36 mg/ml</td>
<td>6</td>
</tr>
<tr>
<td>0.18 mg/ml</td>
<td>11</td>
</tr>
<tr>
<td>0.09 mg/ml</td>
<td>16</td>
</tr>
<tr>
<td>131I-IgG Human anti-AHF</td>
<td></td>
</tr>
<tr>
<td>0.75 mg/ml</td>
<td>4</td>
</tr>
<tr>
<td>0.38 mg/ml</td>
<td>13</td>
</tr>
<tr>
<td>0.19 mg/ml</td>
<td>27</td>
</tr>
<tr>
<td>0.09 mg/ml</td>
<td>31</td>
</tr>
</tbody>
</table>

*One-tenth milliliter of antibody was incubated with 0.1 ml of normal human plasma for 2 hr at 37°C. Residual AHF activity was determined using normal human plasma incubated with an equal volume of barbital-saline buffer as standard.

Properties of this IgG antibody have been reported. This antibody does not form precipitin lines when tested in immunodiffusion assays using normal plasma or AHF concentrates.

The unfractionated human and rabbit sera had similar anti-AHF titers, and the AHF inactivation was time and temperature related to the same degree. A 1:200 dilution of these sera inactivated 40–60% of the AHF activity in an equal volume of normal plasma during a 1-hr incubation at 37°C. The purified and radiolabeled IgG fractions prepared from these sera retained their capacity to inactivate AHF (Table 1). The specific anti-AHF activity of the 125I-IgG

Table 2. Antibody Binding Measured by Shift of Radiolabeled IgG Anti-AHF to the Void Volume on Sepharose 6B Chromatography

<table>
<thead>
<tr>
<th>Rabbit Anti-AHF (0.36 mg/ml)</th>
<th>Normal Rabbit (0.35 mg/ml)</th>
<th>Human Anti-AHF (0.75 mg/ml)</th>
<th>Normal Human (0.70 mg/ml)</th>
<th>AHF Source</th>
<th>% Radioactivity Eluted at Void Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/ml</td>
<td>2 ml Concentrate*</td>
<td>6.90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>2 ml Concentrate</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>2 ml Concentrate</td>
<td>0.18†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>2 ml Concentrate</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mg/ml</td>
<td>0.1 ml Normal plasma</td>
<td>6.58‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mg/ml</td>
<td>0.005 ml Normal plasma</td>
<td>1.82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mg/ml</td>
<td>0.001 ml Normal plasma</td>
<td>0.65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mg/ml</td>
<td>None</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mg/ml</td>
<td>0.1 ml Normal serum</td>
<td>6.90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mg/ml</td>
<td>0.1 ml Hemophilia</td>
<td>8.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mg/ml</td>
<td>A plasma (&lt;1 U AHF/100 ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mg/ml</td>
<td>0.1 ml von Willebrand’s disease plasma (2 U AHF/100 ml)</td>
<td>0.57</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*An AHF-rich concentrate of normal human plasma prepared by precipitation at −3°C with 3% ethanol and dissolved in one-fifth original plasma volume.
†Average of two experiments (0.14, 0.22).
‡Average of two experiments (5.96, 7.20).
rabbit anti-AHF was approximately twice that of the $^{131}$I-IgG human anti-AHF.

Antihemophilic factor may be separated from most other plasma components by agarose gel filtration\(^2\) for it is eluted at the void volume in contrast to smaller proteins like IgG. As any complex of smaller proteins with AHF will also be eluted at the void volume, this separation method is a simple way to detect IgG bound to AHF. As much as 6.9% of $^{125}$I-labeled rabbit IgG was eluted at the void volume when this protein was incubated with AHF prior to gel filtration (Table 2). As these conditions include mixtures with excess antigen, nearly 7% of the total rabbit IgG was anti-AHF (approximately, 0.7 mg anti-AHF/ml rabbit serum). In contrast, only small amounts of $^{131}$I-IgG human anti-AHF were eluted with the void volume fractions (0.14–0.49% in five separate experiments). Almost all of the normal rabbit IgG and normal human IgG were also eluted with the smaller proteins.

The different properties of rabbit and human IgG are clearly apparent in Figs. 1 and 2. Chromatography of 2 ml of an AHF-rich concentrate of normal human plasma incubated with equal amounts of rabbit and human IgG anti-AHF indicates the markedly reduced binding properties of human anti-AHF (Fig. 1). While 6.92% of the rabbit IgG was eluted at the void volume, only 0.22% of the human IgG was shifted into these fractions. The different properties of the two antibodies were also apparent when eight times as much human IgG was used, providing at least fourfold greater AHF-inactivating activity. With this ratio, 7.35% of the rabbit IgG was eluted in the void volume fractions, while only 0.41% of the human IgG was shifted (Fig. 2).

![Fig. 1. Chromatography of 7.2 µg rabbit $^{125}$I-IgG anti-AHF (closed circles), 7.5 µg human $^{131}$I-IgG anti-AHF (open circles), and 2 ml of an AHF-rich concentrate (4.5 U AHF/ml) of normal human plasma on Sepharose 6B. As specific radioactivity of human IgG was 3.3 times that of rabbit IgG, the net cpm for human IgG have been multiplied by 0.3 to allow comparison of equivalent amounts of antibody protein.](image-url)
Fig. 2. Chromatography of 3.6 μg rabbit 125I-IgG anti-AHF (closed circles), 30 μg 131I-IgG human anti-AHF (open circles), and 2 ml of normal human plasma on Sepharose 6B. As specific radioactivity of human IgG was 3.3 times that of rabbit IgG, the net cpm for human IgG have been multiplied by 0.3 to allow comparison of equivalent amounts of antibody protein.

This experiment was repeated using 2 ml of the AHF-rich concentrate in place of normal plasma with similar results (8.80% of rabbit IgG and 0.32% of human IgG eluted in the void volume fractions).

This method provides an extremely sensitive measure of AHF antigens. A shift of radiolabeled IgG to the void volume was detected when as little as 0.001 ml of normal plasma was incubated with the rabbit anti-AHF. As expected from the data that Zimmerman et al. and Stites et al. have published,4,5 normal human serum and hemophilic plasma were as effective in binding the rabbit anti-AHF as was normal human plasma (Table 2). Very little anti-AHF IgG was shifted to the void volume fractions when von Willebrand's disease plasma was tested, however, indicating that there is a marked reduction in AHF antigen as well as AHF activity in this condition. One-tenth milliliter of von Willebrand's disease plasma (0.002 U AHF activity) bound approximately as much rabbit anti-AHF as did 0.001 ml of normal human plasma (0.001 U AHF activity).

Unlabeled anti-AHF was incubated with normal human plasma (0.1 ml) for 30 min at 37°C before the 125I-IgG rabbit anti-AHF (7.2 μg) was added in a series of experiments that established the specificity of the binding. A tenfold excess of unlabeled rabbit IgG (72 μg) markedly reduced the amount of radio-labeled antibody eluted with the void volume fractions; from 7.2 to 3.1% was eluted when 0.1 ml normal plasma was chromatographed. In contrast, a
tenfold excess of unlabeled human IgG anti-AHF had no effect on the elution properties of the labeled rabbit antibody; 7.9% of the radioactivity was eluted in the void volume fractions when 74 μg human IgG was added. If the amount of AHF antigen was limited (0.005 ml normal plasma), a fivefold excess of unlabeled rabbit IgG (36 μg) completely suppressed the binding of 7.2 μg 125I-IgG anti-AHF (from 1.82 to 0.14%).

Additional evidence for the specificity was obtained by the chromatography of the labeled rabbit IgG with an AHF-poor preparation obtained from 0.1 ml of normal plasma (those proteins which were soluble in 3% ethanol but precipitated by 8% ethanol at —3 C). Only 0.32% of the 125I-IgG was eluted with the void volume fractions in this experiment.

DISCUSSION

The recognition of molecular heterogeneity in hemophilia A1-5 and hemophilia B13,15 is based upon immunologic demonstration of nonfunctional, antigenically cross-reacting molecules. Studies using antibodies of human origin have generally indicated that in both diseases there is a subgroup of patients (approximately 10%) in which cross-reacting material may be demonstrated. Experiments using duck16 and goat17 antihuman AHF have given similar results. In contrast to these findings are recent reports that all patients with hemophilia A have cross-reacting AHF-like molecules in their plasmas when they are tested with rabbit anti-AHF.4,5

Taken together, these studies suggest that hemophilia A may be caused by different molecular defects that cause variable loss of antigenicity. While rabbit anti-AHF reacts with all of these molecules, human antibodies are not neutralized by molecules that have extensive changes (and, therefore, loss of antigenic determinants) but only by molecules from the subgroup of patients in which there have been less extensive changes in AHF structure. Stites et al. have noted the similarities between these findings and the studies of human and rabbit antibodies to human IgG. The homologous antibodies have limited reactivity in both instances (related to allotypic differences in the case of IgG) while the heterologous antibodies are uniformly reactive.5

The different findings with circulating anticoagulants and heterologous antibodies have been, therefore, ascribed to differences in the sites on the AHF molecule with which they react.4,5 The competitive binding experiments presented in this paper are consistent with this interpretation but they do not establish these differences. It would be surprising if the weak AHF-human antibody interaction displaced the strongly binding rabbit anti-AHF.

Even if it were established that the two antibodies reacted with different antigenic determinants, other reasons would have to be sought for the differences in secondary properties. The studies that have demonstrated a subunit structure for AHF make it unlikely that human anti-AHF cannot precipitate with AHF because the antigen is univalent.18,19 Our binding studies also establish that AHF is multivalent. (If there is 0.01 mg AHF/ml human plasma4 and its molecular weight is approximately two million,12 anti-AHF binding by 0.005 ml normal plasma (Table 2) indicates at least 35 sites/molecule.) Another basis must be sought, therefore, for the lack of precipitate
formation by potent human anti-AHF with normal plasma or AHF-rich concentrates of normal plasma. Our data establish that the binding properties of human anti-AHF are so weak that a stable complex is not formed. The human anti-AHF can inactivate the procoagulant properties of this molecule even though it does not form a firm linkage.

Rabbit IgG anti-AHF, in contrast, forms stable complexes with the procoagulant and can, therefore, be recognized by precipitation or hemagglutination-inhibition assays. Gralnick et al. have recently noted that a goat antihuman AHF detects cross-reacting material in a small percentage of hemophiliacs and that these patients can be identified by precipitation lines on immunoelectrophoresis of their plasmas, as well as by antibody neutralization assays. This indicates that the difference in secondary properties of rabbit and human antibodies may not be related to the differences in the AHF antigenic sites with which they react.

Agarose gel filtration permits the separation of proteins and complexes with molecular weights greater than one million from smaller molecules. This property has been used to demonstrate the formation of complexes of IgG antibodies (molecules that usually are retarded in agarose gel chromatography) with an antigen that has molecular weight such that it emerges in the void volume. Bound and free IgG are, therefore, effectively separated by agarose gel filtration. While this separation method was developed to study the properties of the different kinds of anti-AHF, it may also prove useful as a method for the detection of very small amounts of AHF antigen (Table 2). There is little doubt that this "radioimmunoassay" for AHF antigen can be modified to provide a rapid means for the measurement of this important protein. An assay for AHF antigen is of value in the clinical differentiation of hemophilia A from von Willebrand's disease and in the identification of hemophilia carriers. A quantitative assay that can be done using small amounts of unconcentrated plasma will simplify studies and will make them more generally available.

ACKNOWLEDGMENT

The expert technical assistance of Mrs. Carol Church is gratefully acknowledged. Dr. Robert Breckenridge kindly provided plasma samples from his patient A.R. and collaborated in the preparation of the rabbit antisera.

REFERENCES

ANTIHEMOPHILIC FACTOR

Immunologic Studies of Antihemophilic Factor (AHF, Factor VIII). III. Comparative Binding Properties of Human and Rabbit Anti-AHF

Leon W. Hoyer