Immunologic Characterization of 12 Factor VIII Inhibitors

By Donald I. Feinstein, Samuel I. Rapaport, and May N. Y. Chong

ACQUIRED INHIBITORS of clotting factors are a well-documented cause of hemorrhagic disease.\(^1\)\(^2\) Inhibitors have been described against Factor I (Fibrinogen), Factor V (Proaccelerin), Factor VIII (Antihemophilic globulin), Factor IX (PTC), and Factor XIII. All of these inhibitors have been characterized as immunoglobulins except for the last.\(^3\) The most common inhibitor has been directed against Factor VIII and has been found in four clinical situations\(^1\)\(^2\):

1) Hereditary Factor VIII deficiency after transfusions.
2) Disorders characterized by abnormal immunologic phenomena, e.g., systemic lupus erythematosus, rheumatoid arthritis, and drug reactions.
3) Post-partum women.
4) Patients without underlying disease.

It has been proposed that these inhibitors are monotypic K\(_{2\gamma 2}\) antibodies.\(^4\)\(^5\) However, there has been a recent report of a \(\lambda_{2\mu 2}\) Factor VIII inhibitor.\(^6\) Also, an IgA myeloma protein has been described with Factor VIII inhibitor activity.\(^7\) Because Factor VIII inhibitors are relatively uncommon, it is difficult to study a large number in one laboratory. Through the cooperation of several investigators, we obtained plasma from 19 patients with a Factor VIII inhibitor.\(*\) This paper is a report of the immunologic characterization of these inhibitors by immune neutralization with specific antisera.

METHODS

1. Clotting Methods

Platelet-poor plasma was prepared by high speed centrifugation of 9 volumes of venous blood added to 1 volume of a balanced citrate anticoagulant.\(^8\) Plastic syringes and tubes were used. Serum was obtained by clotting blood in glass tubes, incubating the clotted blood for 2 hours at 37 C., adding 1 part of citrate anticoagulant for each 9 parts of blood, and allowing the citrated serum to stand for 2 hours more at 37 C. Plasma and serum either used fresh or after storage at \(-20\) C. in capped, plastic vials.

Factor VIII was measured in a one-stage activated partial thromboplastin time assay as described elsewhere.\(^8\)

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\(\text{\(*\)We sincerely thank Drs. Sylvia Hoag, Cecil Hougie, Carol Kasper, Judith Pool, Arthur Seamen and Ralph Wallerstein for plasma samples.}\)
2. Immunologic Methods

Starch zone electrophoresis was performed by the technic of Kunkel,9 chromatography on DEAE by the technic of Fahey and Horbett;10 and chromatography on Sephadex G-200 by the technic of Flodin and Killander.11 IgG was digested with papain and the Fab and Fc fragments were separated by chromatography on DEAE cellulose as described by Franklin.12 Protein concentration was determined either by a modified Folin technic or by measuring absorbancy at 280 m\textmu. Antigens were emulsified with an equal volume of complete Freund's adjuvant to give a final protein concentration of 2.5 mg./ml.

Antihuman immunoglobulin antiserum was prepared by hyperimmunizing rabbits to human Cohn fraction II which had been purified by passage through a DEAE column equilibrated with a phosphate buffer at pH 8.0 and ionic strength .01. The antiserum reacted strongly with K and \( \lambda \) light chains and with \( \gamma \) chains, weakly with \( \alpha \) chains, and not with \( \mu \) chains. Since it reacted with both antigenic types of light chains, it reacted with all classes of human immunoglobulins and was therefore an antihuman immunoglobulin antiserum.

An antiserum specific for IgG was made by absorbing the above antiserum with purified Fab fragment which had been prepared by papain digestion of purified Cohn fraction II and subsequent isolation of the Fab and Fc fragments on DEAE cellulose.

Antiserum specific for IgM was made by hyperimmunizing rabbits with a macroglobulin isolated from the serum of a patient with macroglobulinemia of Waldenström by preliminary dialysis against distilled water followed by G-200 Sephadex chromatography. This material gave a single line on immunoelectrophoresis with rabbit antihuman serum. The antiserum obtained with this antigen was absorbed with umbilical cord sera to make it specific for \( \mu \) chains.

Antiserum specific for IgA was prepared by hyperimmunizing rabbits with an IgA myeloma protein which was isolated by starch block electrophoresis and further purified by column chromatography on Sephadex G-200. Eluates from the first half of the first peak were combined and concentrated. This material contained less than 1 per cent IgG. The antiserum obtained with this antigen was absorbed with umbilical cord sera to make it specific for \( \alpha \) chains.

Antisera specific for K and \( \lambda \) light chains were prepared by hyperimmunizing rabbits to K and \( \lambda \) Bence-Jones proteins isolated from the urine of patients with multiple myeloma by precipitation with 40 per cent ammonium sulfate followed by starch zone electrophoresis. The antisera were made specific by absorption with the opposite antigenic type of Bence-Jones protein, with IgG myeloma protein and with Fc fragment.

Clotting factor activities were removed from the antisera and from normal rabbit sera as follows: 1 part of citrate anticoagulant was added to 5 parts of serum; the serum was heated at 56 C. for 2 hours; and the heated serum was then adsorbed with an equal volume of A1(OH)\textsubscript{3} gel (Cutter Laboratories, Berkeley, California) diluted 1:4 with distilled H\textsubscript{2}O. After such treatment all sera were devoid of clotting factor activities which would interfere with the Factor VIII assay.

To interpret the neutralization experiments the zone of antigen and antibody excess for each reaction mixture had to be defined. The reaction mixtures consisted of the patient's plasma containing the inhibitor (antigen) and the immunoglobulin antiserum (antibody). Therefore, the patient’s plasma was diluted in increasing amounts of each immunoglobulin antiserum; incubated at 37 C. for 1 hour, centrifuged; and the supernatant was reacted in double diffusion in agar against the patient’s plasma (antigen) and the antiserum (antibody). If the supernatant reacted with the antiserum but not with the patient’s plasma, then the dilution was in antigen excess. If the supernatant reacted with the patient’s plasma but not with the antiserum, then the original dilution was in antibody excess. If the supernatant reacted with neither the patient’s plasma nor the antiserum, then the dilution was near the equivalence point.

Immune neutralization was then determined as illustrated in Figure 1. In Mixture #1 the patient’s plasma was diluted either in antiserum to equivalence or antibody excess or in normal rabbit serum (control) and was incubated at 37 C. for 1 hour. After centrifuga-
IMMUNOLOGIC CHARACTERIZATION

Mixture #1 -- Inhibitor plasma
+ Antiserum at equiv.
  inc. at 37°C
  for 1 hr, centrifuge
  Mixture #2 -- Supernatant of Mix. #1
  + Equal vol. of normal pl.
    inc. at 22°C
    for 90'
  Assay for Residual Factor VIII

Fig. 1.—Technic for evaluating the immune neutralization of Factor VIII inhibitor.

The supernatant of Mixture #1 was added to an equal volume of normal plasma. This mixture (Mixture #2) was incubated for 90 minutes at 22°C and its residual Factor VIII activity was measured. If the inhibitor were completely neutralized by the antiserum in Mixture #1, then there would be no significant loss of Factor VIII activity in Mixture #2. In contrast, if the inhibitor were not neutralized by the antiserum, then the Factor VIII activity in Mixture #2 would not significantly exceed that observed in the control.

RESULTS

Nineteen Factor VIII inhibitors were studied. Twelve could be characterized whereas 7 could not because of relatively low titers of the inhibitor. The data are summarized in Tables 1 and 2.

As Table 1 shows, a high residual Factor VIII activity, significantly exceeding that found in the control mixture, was observed in all 12 patients when inhibitor plasma was mixed with polyvalent immunoglobulin antiserum. Therefore, all of the inhibitors were immune globulins. When inhibitor plasma was incubated with class specific antisera, high residual Factor VIII activity was found in the IgG antiserum mixtures, whereas the residual Factor VIII activity for the IgA and the IgM antiserum mixtures was not significantly different from the controls. Therefore, all of the inhibitors were immunoglobulins of the IgG class.

The data on light chain types are summarized in Table 2. The first 10 inhibitors were completely neutralized by the K chain antiserum but were not significantly affected by the \( \lambda \) chain antiserum. In contrast, the last two inhibitors were neutralized by the \( \lambda \) chain antiserum but were not significantly affected by the K chain antiserum. Therefore, 10 inhibitors had K chain determinants and 2 inhibitors had \( \lambda \) chain determinants. The two \( \lambda_{27\gamma} \) in-
Table 1.—Determination of Immunoglobulin Class of Factor VIII Inhibitors

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>Normal Rabbit Serum</th>
<th>Anti Immunoglobulin AS IgG</th>
<th>Anti IgA</th>
<th>Anti IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo</td>
<td>21%</td>
<td>90%</td>
<td>60%</td>
<td>24%</td>
</tr>
<tr>
<td>Fo</td>
<td>0%</td>
<td>83%</td>
<td>83%</td>
<td>0%</td>
</tr>
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<td>En</td>
<td>10%</td>
<td>70%</td>
<td>66%</td>
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<td>Mn</td>
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<tr>
<td>Ar</td>
<td>32%</td>
<td>80%</td>
<td>78%</td>
<td>32%</td>
</tr>
<tr>
<td>Le</td>
<td>32%</td>
<td>76%</td>
<td>84%</td>
<td>22%</td>
</tr>
<tr>
<td>Co</td>
<td>25%</td>
<td>100%</td>
<td>100%</td>
<td>26%</td>
</tr>
<tr>
<td>No</td>
<td>44%</td>
<td>110%</td>
<td>150%</td>
<td>44%</td>
</tr>
<tr>
<td>Ha</td>
<td>15%</td>
<td>90%</td>
<td>90%</td>
<td>15%</td>
</tr>
<tr>
<td>Ob</td>
<td>11%</td>
<td>54%</td>
<td>76%</td>
<td>16%</td>
</tr>
<tr>
<td>Go</td>
<td>19%</td>
<td>74%</td>
<td>120%</td>
<td>17%</td>
</tr>
<tr>
<td>Cl</td>
<td>30%</td>
<td>80%</td>
<td>86%</td>
<td>25%</td>
</tr>
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</table>

The patient's plasma was incubated with each antiserum at, or near, equivalence for 60 minutes at 37°C. The precipitate was centrifuged, the supernatant was incubated with an equal amount of normal plasma for 90 minutes at room temperature, and residual Factor VIII activity was measured.

Table 2.—Determination of Light Chain Type of Factor VIII Inhibitors

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>Normal Rabbit Serum</th>
<th>Anti K</th>
<th>Anti γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo</td>
<td>31%</td>
<td>100%</td>
<td>31%</td>
</tr>
<tr>
<td>Fo</td>
<td>12%</td>
<td>76%</td>
<td>16%</td>
</tr>
<tr>
<td>En</td>
<td>12%</td>
<td>90%</td>
<td>14%</td>
</tr>
<tr>
<td>Mn</td>
<td>26%</td>
<td>68%</td>
<td>33%</td>
</tr>
<tr>
<td>Ar</td>
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<td>100%</td>
<td>30%</td>
</tr>
<tr>
<td>Le</td>
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<td>72%</td>
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</tr>
<tr>
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<td>110%</td>
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<td>92%</td>
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<td>90%</td>
<td>17%</td>
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<td>Ob</td>
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<td>58%</td>
<td>13%</td>
</tr>
<tr>
<td>Go</td>
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<td>17%</td>
<td>74%</td>
</tr>
<tr>
<td>Cl</td>
<td>28%</td>
<td>33%</td>
<td>56%</td>
</tr>
</tbody>
</table>

* See footnote to Table 1.

Our results support the existing evidence that Factor VIII inhibitors are immunoglobulins that are homogeneous in regard to immunoglobulin class and light chain type by the method of immune neutralization. However, all of these inhibitors are not of the K2γ2 type.4,5

Discussion

Our results support the existing evidence that Factor VIII inhibitors are immunoglobulins that are homogeneous in regard to immunoglobulin class and light chain type by the method of immune neutralization. However, all of these inhibitors are not of the K2γ2 type.4,5
The literature contains data on 14 other Factor VIII inhibitors that have been immunologically characterized, including light chain typing. Fourteen of these have been IgG immunoglobulins and 1 has been IgM. The 13 IgG inhibitors contained only K chain determinants; the IgM inhibitor contained only λ chain determinants. Also, an IgA myeloma protein has been described with Factor VIII inhibitor activity. One of the Factor VIII inhibitors with K2y2 determinants has also been characterized as to H-chain subtype. Interestingly, this inhibitor only had γG4 antigenic determinants and no detectable γG1, γG2, or γG3 antigenic determinants.

There is evidence that a given human lymphoid cell synthesizes γG globulin molecules with only one of 2 possible light chain types and one of 4 possible heavy chain subtypes. Myeloma proteins have only 1 light and 1 H-chain subtype which is thought to reflect their synthesis by a single cell clone. Many normal human antibodies and several types of autoantibodies have been found to contain both K and λ light chain types in the approximate ratio that they are found in normal human serum. Presumably, therefore, these antibodies have been made by more than one clone of cells. However, several autoantibodies have been shown to have a marked predominance of K or λ type molecules, and, in some instances, have been as homogeneous as myeloma proteins. Apparently, isolated human antibodies may be either homogeneous or exhibit a wide spectrum of heterogeneity.

The findings for Factor VIII antibodies suggest that these antibodies resemble myeloma proteins in that they are a single class of immunoglobulins with a single light chain type and, in the one case studied to date, with a single H-chain subtype.

This hypothesis, however, must be viewed with caution, in light of the experience with the immunologic characterization of red cell autoantibodies. These antibodies also were originally thought to have homogeneous light chain determinants, but further study with H-chain subtyping antisera and more sensitive immunochemical technics have shown them to have variable heterogeneity.

Thus, although all 26 Factor VIII antibodies that have been characterized to date have been homogeneous, a definitive conclusion cannot be made until more H-chain subtyping can be carried out.

**Summary**

Twelve Factor VIII inhibitors were characterized as immunoglobulins by highly specific antisera. All 12 inhibitors were characterized as belonging to the γG class of immunoglobulins. Ten inhibitors contained only K type light chain determinants and two contained only λ chain determinants. Therefore, these inhibitors were homogeneous by these criteria. Homogeneity cannot be established, however, until H-chain subtyping can be carried out.

**SUMMARIO IN INTERLINGUA**

Dece-duo inhibitores de Factor VIII esseva characterisate como immunoglobulinas per le uso de altemente specific antiseros. Omne le 12 inhibitores esseva characterisate como pertinente al classe γG de immunoglobulinas. Dece inhibitores contineva exclusivemente determinantes a catena leve del typo K, e duo contineva exclusivemente determinantes a
Per consequente, iste inhibitores esseva homogenee secundo le criterios usate. Tamen, il non es possibile establir le homogeneitate ante que subtypification de catena H poter esser effectuate.

REFERENCES

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