Antibodies to Factor VIII. I. Variations in
Stability of Antigen–Antibody
Complexes in Hemophilia A

By J.-P. Allain and D. Frommel

Human factor VIII-anti-factor VIII complexes were formed in vitro in slight antigen excess, using plasma of hemophiliacs who were found to have antibodies neutralizing AHF activity. These complexes, stable at +37°C and pH 7.4, were submitted to classical procedures known to favor dissociation of antibody from antigen. The methods used to obtain dissociation, incubations at +56°C and at pH 4.2, inactivated both unbound factor VIII and that released as a consequence of dissociation.

The extent of dissociation was measured by the recovery of anti-factor VIII activity. Increasing resistance of complexes towards dissociation was observed in the plasma of the patients whose titer of inhibitor was increasing after recent transfusions. These observations suggested the emergence, as a direct consequence of renewed antigenic stimulation, of a population of different antibodies characterized by higher combining strength.

Comparisons between the mode of action of antibodies neutralizing factor VIII and antibodies neutralizing viruses have been recently underlined by Pool and Miller. These analogies were mainly supported by similar kinetics of inactivation.

Human antibodies to antihemophilic factor (AHF) exhibit a number of properties which distinguish them from the antibodies described for the classical pattern of humoral immune response: molecular uniformity, absence of precipitin and complement-fixing properties, and low binding towards antigen. Although some of these characteristics might be explained by the low degree of immunogenicity of isologous antigens, it appeared of importance to analyze the dynamics of this anti-factor VIII response in terms of its combining power towards its antigen. This parameter can indeed be taken as an indirect measure of antibody avidity or affinity, a factor which best reflects the adaptation of the host in the immune response.

Plasma of eight hemophiliacs with various titers of inhibitor were assayed and analyzed with regard to the stability of specific antigen-antibody complexes which had been formed in vitro or occurred in vivo.

Materials and Methods

Blood was collected in uncoated plastic tubes using 0.13 M sodium citrate as anticoagulant (1 part of anticoagulant for 9 parts of venous blood). Samples were centrifuged for 1 hr at +4°C.

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(5,500 g); platelet poor plasma was absorbed with aluminium hydroxide (0.1 ml of a 25° c Al (OH)₃ solution for 1 ml of plasma) for 5 min at +37°C. Plasma samples were stored at −20°C in capped polystyrene tubes.

**Assay of Factor VIII**

A one-stage method¹¹ in presence of kaolin cephalin was used, a citrated plasma of severe hemophiliacs supplying all clotting factors, except factor VIII. The reference plasma for factor VIII assays was a pool of citrated, absorbed, plasma from three healthy male blood donors. One unit of antihemophilic factor was defined arbitrarily as that contained in 1 ml of plasma.¹² All dilutions were performed with veronal acetate buffer.

**Inhibitor Assay**

The method initially described by Biggs and Bidwell¹³ was modified.¹⁴,¹⁵ All plasmas with inhibitor were diluted with buffer to contain approximately 1 unit of anti-factor VIII antibody. One unit of antibody was defined as the amount of antibody which inactivated, after 2 hr of incubation at +37°C, 75% of the factor VIII present in an equal volume of normal plasma (ratio inhibitor/normal plasma 1:1). The residual factor VIII activity was measured in duplicate by the one-stage method, and the titers of the inhibitors were determined using the dilutions of neutralizing plasmas at which 10–30% of factor VIII remained active.

**Influence of Temperature and pH on Factor VIII Activity and on Antibody to Factor VIII**

Normal plasma was incubated for 30 min at +37, 50, 56, and 60°C. After cooling and centrifugation, factor VIII activity was tested in the supernatant. Normal plasma was adjusted to pH ranging from 2.5 to 9.0. The pH was adjusted by slow addition of either 0.1 N HCl or 0.1 N NaOH. After incubation at +37°C for 30 min, the plasma was neutralized, cooled, and brought to a constant final volume and factor VIII assay performed. The same procedures were applied to plasma displaying inhibitor activity. After pH adjustment and centrifugation, inhibitor assay was performed on the supernatant.

**Source of Anti-Factor VIII Antibodies**

Citrated plasma from eight severe hemophiliacs who were found to have inhibitor to factor VIII were studied. "AHF-like antigen," as detected by a rabbit antiserum specific for human factor VIII,¹⁵ was present in their plasma. Patients 1, 2, 6, 7, and 8 were treated with plasma and human blood derives only. Patient 3, also multitransfused, received one course of treatment with porcine AHF 2 yr before analysis. The patients 1, 2, 3, as well as 6 and 7 at the time when their plasma (a) was studied, were in a steady state, as defined by the absence of transfusions for 1 yr or more and stable titer of anti-factor VIII activity. In addition to human cryoprecipitate, patients 4 and 5 were stimulated on one occasion by AHF of animal origin: patient 4 with porcine and afterwards with bovine AHF 3 mo before study, and patient 5 with porcine AHF 6 mo prior to analysis. Samples of three patients were drawn before (6a, 7a, and 8a) and after (6b, 7b, and 7c, 8b and 8c) transfusions with human cryoprecipitate. Plasma 6(b) was obtained 12 days after a single transfusion of cryoprecipitate from an individual donor, equivalent to 750 ml of plasma. One specimen was obtained from patient 7 during continuous infusion of cryoprecipitate, which was requested by a life-threatening hemorrhage. At this time (cf. Table 2, below), the inhibitor was saturated and factor VIII activity could be maintained above 10% (antigen excess). Blood sample 8(a) was analyzed seven months after a stimulation with cryoprecipitate, following which antibody titer peaked at 200 U.

**Stability of the Antigen-Antibody Complexes Formed in Slight Excess of Factor VIII**

A modification of the method outlined by Green¹⁶ was employed. The dilutions of antibody used were such that, after incubation with normal plasma at +37°C, the activity of residual factor VIII remained stable within the range of 10–30% (slight antigen excess) when tested 2, 4, and 6
hr later. Inhibitory plasmas, at these dilutions, were incubated for 2 hr at +37°C with normal plasma; these mixtures were then divided into two aliquots. Residual factor VIII was measured in one part and to the other an equal volume of reference plasma was added (large antigen excess: 60-80% of factor VIII). Factor VIII was assayed in this second mixture at the time of and after 1 and 2 hr of incubation at +37°C. Factor VIII activity of these mixtures was expressed in relation to that of normal plasma diluted with buffer and incubated likewise. No inactivation of factor VIII during and after the second incubation was detected. Thus it could be assumed that, in these conditions, all antibodies to AHF were bound and that stable complexes had been formed.

**Dissociation of Factor VIII–Anti-Factor VIII Complexes by Changes in Temperature and pH**

Mixtures of normal plasma and inhibitors, at the dilutions found to allow formation of stable complexes, were incubated for 2 hr at +37°C, or 2 hr at +37°C followed by 14 hr at +4°C. At the end of these incubations, the samples were incubated for 30 min either at +56°C or at pH 4.2, as described for antigen and antibody. After cooling or pH neutralization followed by centrifugation, the presence of anti-factor VIII antibody, released by these procedures, was sought in the supernatant. The method employed was that of the inhibitor assay, and the residual factor VIII was determined for duplicates of at least two different dilutions of the mixture (supernatant with normal, untreated, plasma).

**RESULTS**

Inactivation of factor VIII following incubations at pH outside the range of 6.0-8.0 or above +50°C illustrated the structural lability of those biologically active molecules (Fig. 1). These denaturative effects could be shown to affect directly factor VIII activity and were not as a result of coprecipitation with fibrinogen. This was demonstrated by submitting plasma from congenital afibrinogenemia to similar procedures: 30 min of incubation at +56°C inactivated 95% ± 3% of factor VIII (mean of four determinations) and at pH 4.2 95% ± 5%. The thermal stability and resistance to acid pH of the human anti-factor VIII antibodies exceeded, as expected, those of the procoagulant proteins (Fig. 1).

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**Fig. 1.** Comparison of pH (A) and temperature (B) dependence between human factor VIII activity (normal plasma - ○) and antibodies to factor VIII of hemophiliacs (mean of 3 antibodies - ■). Following 30 min of incubation, cooling or adjusting to pH 7.3, and centrifugation, residual factor VIII activity or inhibitor assay were performed. Factor VIII activity is expressed in percent of normal, nonincubated, plasma.
Thus it was possible to define conditions, known to favor dissociation of pre-formed antigen-antibody complexes, in which antibody function would remain unaltered but in which the antigen, the factor VIII, present in excess or released during the subsequent procedures, would be inactivated: 30 min at +56°C or at pH 4.2. With plasma of patients displaying stable inhibitory activities to factor VIII for 1 yr or more (cases 1, 2, 3, 6a, and 7a), it was possible to recover from the incubation mixture, either by incubation at 56°C or at pH 4.2, 10%-45% of the anti-factor VIII activity. Prolongation of incubation of antigen-antibody mixtures at +4°C resulted in lower recovery of antibody activity in most instances (Table 1).

When comparing the data obtained with cases No. 6 and 7 before and after restimulation with human blood derivatives, a strong increase in the resistance of dissociation of the factor VIII-anti-factor VIII complexes was apparent in the samples obtained following reimmunization (6b, 7b, and 7c), since little or no antibodies could be recovered. The analysis of case No. 8 which has also been studied under the same conditions, led to similar observations. Between the determinations 7(a) and 7(b), at the time of replacement therapy, it was possible to demonstrate, although circulating factor VIII was in excess, the existence of the underlying inhibitor by incubation at pH 4.2. A partial dissociation with recovery of 0.7 U of inhibitor was observed (Table 2).

Structural changes of factor VIII molecules, resulting from these processes of mild denaturation, were also revealed by the loss of their immunological reactivity towards corresponding antibodies. In neutralization assays, molecules of human factor VIII from plasma previously heated or acidified neither bound their specific and isologous molecules nor competed with native molecules in

### Table 1. Recovery of Anti-Factor VIII Activity after Various Conditions of Incubation and Following Dissociation Procedures by Heat and Acid

<table>
<thead>
<tr>
<th>Patients</th>
<th>Inhibitor Titer</th>
<th>Incubation 2 hr at +37°C</th>
<th>Dissociation 30 min at +56°C</th>
<th>30 min at pH 4.2</th>
<th>2 hr at +37°C + 14 hr at +4°C</th>
<th>30 min at +56°C</th>
<th>30 min at pH 4.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 V.S.</td>
<td>6 ± 1</td>
<td>2.7 (38%)</td>
<td>1.0 (20%)</td>
<td>0 (0%)</td>
<td>0.6 (12%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 J.D.</td>
<td>5 ± 1</td>
<td>1.0 (22%)</td>
<td>0.4 (9%)</td>
<td>1.5 (27%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 A.C.</td>
<td>5 ± 1</td>
<td>1.3 (21%)</td>
<td>1.0 (18%)</td>
<td>1.5 (21%)</td>
<td>1.2 (20%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 G.Y.</td>
<td>30 ± 2</td>
<td>14.0 (31%)</td>
<td>6.0 (20%)</td>
<td>1.3 (5%)</td>
<td>3.6 (12%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 B.L.</td>
<td>20 ± 1</td>
<td>0 (0%)</td>
<td>4.0 (21%)</td>
<td>0 (0%)</td>
<td>1.1 (5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 J.M.C. (a)</td>
<td>5 ± 1</td>
<td>1.2 (31%)</td>
<td>1.8 (47%)</td>
<td>0 (0%)</td>
<td>0.3 (8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) 1.225 ± 100</td>
<td>80 (7%)</td>
<td>50 (4%)</td>
<td>NT</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 L.L. (a)</td>
<td>4 ± 1</td>
<td>3.2 (66%)</td>
<td>0.7 (23%)</td>
<td>0.2 (4%)</td>
<td>0.3 (1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) 22 ± 3</td>
<td>1.5 (7%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c) 50 ± 6</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 J.C.L. (a)</td>
<td>13 ± 1</td>
<td>0 (0%)</td>
<td>2.6 (22%)</td>
<td>0 (0%)</td>
<td>1.2 (8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) 28 ± 2</td>
<td>0 (0%)</td>
<td>1.5 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c) 170 ± 20</td>
<td>10.0 (5%)</td>
<td>NT</td>
<td>0 (0%)</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The titer given in the second column represents the mean of the determinations performed concurrently with the four tests of dissociation. The titer of the antibody recovered by dissociation is expressed in U and percent of the individual and corresponding determination performed simultaneously with the dissociation assay. The last transfusions of patients 1, 2, 3, 6(a), and 7(a) took place over 1 yr ago.
NT: not tested.
Table 2. Recovery of Anti-Factor VIII Antibody during Replacement Therapy

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Antibody Titer (U)</th>
<th>Factor VIII Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>56°C, 30 min</td>
<td>0.16</td>
<td>&lt;1</td>
</tr>
<tr>
<td>56°C, 60 min</td>
<td>0.22</td>
<td>&lt;1</td>
</tr>
<tr>
<td>pH 4.2, 30 min</td>
<td>0.13</td>
<td>&lt;1</td>
</tr>
<tr>
<td>pH 4.2, 60 min</td>
<td>0.70</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Recovery of anti-factor VIII antibody in a plasma from patient 7 when under continuous infusion of human cryoprecipitate. The titer of the inhibitor determined before replacement therapy was 9 U.

the reaction of neutralization (Table 3). However, heteroantibodies, in this instance a specific rabbit antiserum, still interacted with these biologically inactive molecules giving precipitin reactions in gel (unpublished data).

DISCUSSION

Earlier studies dealing with lability of human factor VIII showed that biological activity of factor VIII decreased rapidly outside the pH range 6.5–8.0 and above +45°C. Our results obtained from studies of normal and afibrinogenemic plasma confirmed these previous findings and proved that inactivation of AHF was due to elevations in temperature or changes in pH, and was not dependent upon modifications undergone by fibrinogen. Since heating or acidification, while destroying free antigen, spared antibody activity, it was possible to study patterns of dissociation in following recovery of antibody. The amount of dissociated antibody could be measured according to the inactivation observed after the addition of an aliquot of untreated plasma.

Methods of shifting equilibrium of immune complexes towards dissociation, such as elevation in temperature, lowering of pH, and increase of ionic strength, affect the conformations of the antigen and the antibody and loosen their intermolecular bonds. Since resistance to dissociation is directly correlated to the degree of mutual complementarity between the two reactants, antibodies

Table 3. Consumption of Human Anti-Factor VIII Antibody by Normal and Denatured Plasma

<table>
<thead>
<tr>
<th>Factor VIII Activity (%)</th>
<th>Inhibitor Neutralized (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Plasma, undiluted</td>
<td>100</td>
</tr>
<tr>
<td>1:2</td>
<td>51</td>
</tr>
<tr>
<td>1:4</td>
<td>24.3</td>
</tr>
<tr>
<td>Normal Plasma Heated 30 min at 56°C</td>
<td>1.2</td>
</tr>
<tr>
<td>Normal Plasma Acidified</td>
<td>30 min at pH 4.2</td>
</tr>
<tr>
<td>Buffer</td>
<td></td>
</tr>
</tbody>
</table>

Neutralizing activity of anti-factor VIII activity by normal plasma, at three dilutions, and normal plasma treated for 30 min at +56°C or acidified for 30 min at pH 4.2. The inhibitory plasma from patient 7 was diluted 1:5. The technique used by Meyer and Larrieu was slightly modified in that the two incubations were performed for 2 hr at +37°C.
of high affinity require more drastic conditions to reverse antigen-antibody association.

In our protocol, arbitrary conditions were chosen in which the antigen would be preferentially modified. In complexes characterized by weak cohesion force the antigen was either unprotected, inactivated, and released or directly cleaved from the antibody. Whatever is the exact mechanism responsible for dissociation, reactivation of antibody activity may be taken as a parameter of affinity and allows comparison between different inhibitors.

Antibodies obtained from plasma of patients whose last immunization was 1 or more yr before and possessing low titer of inhibitor, showed a lower combining power towards factor VIII than antibody present in plasma from patients who had had more recent transfusions. Indeed, dissociation procedures remained unsuccessful in cases No. 6, 7, and 8, when studied after a restimulation. Prolonged incubation at +4°C, favoring secondary stabilizing reactions, increased the firmness of complexes formed with plasma of patients in a steady state. The finding, during replacement therapy and in presence of measurable factor VIII, of anti-factor VIII activity revealed by dissociation at pH 4.2 confirmed the occurrence of circulating complexes in vivo. This low recovery of antibody, in a plasma which had a titer of 9 U of inhibitor just before the beginning of specific therapy, was of the same order of magnitude as that observed with sample 8(a).

Although this part of the study was limited to only three patients, the demonstration of different properties in plasma obtained before and at intervals after stimulation showed that the immune response to factor VIII in hemophiliacs was characterized not only by an increase in the amount and in the number of specific antibodies but also by the development of different types of antibodies. The first of these two arguments is supported by the fact that, upon appropriate dilution of inhibitor, saturation of anti-factor VIII antibodies from hemophiliacs can always be reached in vitro. The number of antibody molecules thus increased proportionally to the titer of the inhibitor. The second point, already suggested by differences in the kinetics of neutralization, is confirmed in the experiments reported herein which demonstrated an increase in the antibody binding affinity in plasma with high titer, indicating the emergence of antibodies having a better conformational correspondence with the antigen. Therefore antibody formation to isologous factor VIII in hemophiliacs does not differ significantly from the classical pattern of humoral immune response: Antibodies resulting from repeated exposures to antigen express increasing affinity. Some peculiarities are, however, distinctive: the absence of a detectable primary IgM response, and the oligo- or monoclonal character of the specific antibodies. We would suggest that in a steady state, more than one clone synthesizes antibodies differing in specificities. Depending on the conditions of restimulation, the clone with the highest affinity would capture preferentially the antigen, proliferate, and lead to synthesis and secretion of a single predominant type of antibody.

Pool and Miller pointed out the analogies existing between the mode of action of antibodies neutralizing factor VIII and antibodies neutralizing infectivity of viruses. Our results validate and further extend this comparison. Dis-
Fig. 2. Kinetics of antigen-antibody dissociations under acidic conditions as a function of time. (A) Polio virus-rabbit antipolio virus antibody system. Extent of dissociation at pH 4.6 is expressed by reactivation of polio virus infectivity (antigen activity). Adapted from Mandel: Virology 14:323, 1961. (B) Human factor VIII-human anti-factor VIII system. In this situation, the extent of dissociation following incubation at pH 4.2 is measured by the recovery of AHF neutralizing activity (antibody activity). Patient 4, Patient 5, Patient 1.

Association of factor VIII-anti-factor VIII complexes as a function of pH resulted in patterns that were superimposable with those obtained by Mandel24 in a study of the reversibility of interactions between poliovirus and rabbit antiserum (Fig. 2). In man, Brunner and Ward25 have reported that convalescent phase antibody formed complex with poliovirus which upon dilution dissociated at a slower rate than early or acute-phase antibody-virus complexes.

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