Antibodies to Factor VIII. V. Patterns of Immune Response to Factor VIII in Hemophilia A

By Jean-Pierre Allain and Dominique Frommel

The natural history of factor VIII antibodies was studied in 20 severe, multitransfused hemophiliacs. Two patterns of humoral immune reactivity were observed. In one group of ten, who developed antibodies after an average of 22 cumulative exposure days to factor VIII, the antibody titers increased after each antigenic stimulation or persisted for years in the absence of transfusion. These patients were designated as high-responding hemophiliacs. In the second group of ten patients, the factor VIII neutralizing activity appeared after a longer exposure period (48 days). Antibody titers remained low, and there was no significant difference in individual titers before and 8–20 days following transfusion. Antibody affinity did not increase after renewed antigenic challenge. This pattern characterized low-responding hemophiliacs. The latter group of patients benefited from repeated replacement therapy required by the clinical situation.

In severe hemophilia A, antibodies to factor VIII occur in 5%–21% of the patients who receive repeated transfusions of blood or factor VIII containing plasma derivatives.1,2 It is generally accepted that once hemophiliacs are immune to factor VIII further transfusion triggers a stereotyped response. After a lag phase of 5–7 days, the antibody titer increases rapidly reaching a peak 8–20 days poststimulation. When antigenic stimulation is discontinued, the antibody titer most often declines progressively.1,4-6 The properties of these antibodies have been described.7,8

Previous reports have mentioned a few cases of hemophiliacs who displayed an atypical pattern of immune response to factor VIII.1,6,9 These patients are characterized by a limited capacity to produce antibodies to factor VIII despite numerous antigenic stimulations. In this communication, we describe ten additional patients with severe hemophilia A whose factor VIII antibody synthesis consistently remained minimal. The natural history of the development of antibodies in these patients by both in vivo and in vitro studies is compared to ten patients who developed antibodies corresponding to what is classically described as the "typical" immune response to factor VIII.

PATIENTS

The 20 hemophiliacs described in this report were boarded for 2 yr or more at the Center for Hemophilic Children. Each of them had a severe factor VIII deficiency. Prior to admission, five patients were known to have antibodies to factor VIII (cases 11, 13, 15, 19, and 20). Antibodies in the other patients were discovered after they received treatment at the Center. Six patients...
had had replacement therapy for prolonged periods of time as required by surgery or prophylactic treatment (patients 1, 3, 7, 10, 11, and 20).

A systematic program for detection of an immune response to factor VIII has been carried out since 1971. First, after each patient’s series of five transfusions, the in vivo recovery of factor VIII activity was determined. Second, each patient’s plasma was screened in vitro 8-20 days post-transfusion for detection of antibodies to factor VIII.

MATERIALS AND METHODS

Sample Collection and Storage

Blood was collected in plastic tubes containing 0.13 M sodium citrate. The ratio of anticoagulant to blood was 1:9. Samples were centrifuged at 4°C for 1 hr at 5500 g. Platelet-poor plasma was adsorbed with aluminum hydroxide gel (British Drug House, Poole, England) for 5 min at 37°C using 0.1 ml of a 25% suspension per ml of plasma. After centrifugation at 4°C for 10 min at 5500 g, the adsorbed plasma was removed and stored in capped polystyrene tubes at −20°C for no longer than 8 days for factor VIII assays and up to 2 yr for antibody studies.

Factor VIII Assay

A kaolin-activated one-stage factor VIII assay was used.

Factor VIII Recovery and Half-life In Vivo

Factor VIII activities were determined in plasma samples collected at intervals following the infusion of a single dose of 15-25 units of factor VIII per kg of body weight. The source of factor VIII was lyophilized cryoprecipitate (Centre National de Transfusion Sanguine, Paris, France). The amount of factor VIII to be injected was calculated according to the manufacturer’s indications, which proved to be reliable. In a previous study, the observed level of factor VIII determined 30 and 60 min postinfusion gave a valid estimate of the recovery of the infused factor VIII. In the absence of antibody, one unit of infused factor VIII per kg of body weight raised the recipient’s factor VIII level by 0.02 U/ml. Additional controls were performed in a group of 16 hemophiliacs ranging from 5 to 15 yr with an average body weight of 30 kg. The percent of expected recovery was calculated according to the following formula:

\[
\text{% of expected recovery} = \frac{\text{observed recovery (U factor VIII/ml)}}{\text{expected recovery (U factor VIII/kg x 0.02)}}
\]

At the time of sample collection, none of the subjects (control or patient) were febrile or had massive blood loss.

The intravascular half-disappearance time of factor VIII activity was calculated from four determinations of factor VIII obtained in plasma samples collected 4, 8 or 12, 24, and 48 hr post-infusion. Results were plotted on semilogarithmic graph paper.

Antibody Titration

Antibody titration was performed according to a previously described method. The source of antigen was a freshly prepared pool from normal human plasmas adsorbed with aluminum hydroxide gel. Test plasma or its dilutions were mixed with undiluted normal plasma, incubated at 37°C for 2 hr, and tested for residual factor VIII. 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 incubated under the same conditions.

For titration of weak antibodies a more sensitive method was developed. Each antibody sample was concomitantly tested at three antigen-antibody ratios. To 1 volume of normal plasma was added 1, 2, and 3 volumes of undiluted antibody containing plasma. After incubation, the residual factor VIII of each mixture was assayed and expressed as the per cent activity of appro-
proportionately diluted normal reference plasma, similarly incubated. The antibody titer read from the graph of Biggs and Bidwell was multiplied by the antigen-antibody ratio. The effective antibody titer was the mean of the three results. Control tests were performed by a blind procedure and in duplicate on 10 aliquots of the same antibody-containing sample; titers determined with antigen antibody ratios in decreasing order were \(0.65 \pm 0.17, 0.51 \pm 0.06,\) and \(0.57 \pm 0.12\) U/ml, resulting in an average titer of 0.58 U/ml. This technique of titration was valid only if a given plasma sample was tested at the three antigen-antibody ratios. The lowest threshold of reliable antibody titer measurement was 0.2 U/ml.

**Prolonged Incubation of Antigen–Antibody Mixtures**

Plasmas containing antibodies were incubated up to 6 hr at 37°C with either human, porcine (large white strain), or bovine (Charolais strain) Al(OH)₃ adsorbed plasmas. Residual factor VIII was determined at 1-hr intervals. Controls consisted of human, porcine, or bovine plasmas mixed and incubated with hemophilic plasma that did not contain an antibody. Dissociation of antigen-antibody complexes by heat were performed as previously described.

**RESULTS**

**Immunization Patterns Toward Factor VIII**

Patients 1–10 (Table 1) had low titer antibodies, ranging from 0.25 to 3.6 U/ml. These antibodies developed after 18–121 cumulative exposure days (mean 48 days). Since detection of the antibody, most of these patients have been treated repeatedly with no significant increase of the antibody titer. After a prolonged period without stimulation (3–13 mo), the antibody became unde-

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**Table 1. Immunologic Reactivity of High and Low Responders**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at Discovery (yr-mo)</th>
<th>Cumulative Exposure Days</th>
<th>Antibody Titer (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>Since Discovery</td>
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<tr>
<td>Low responders</td>
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<tr>
<td>1</td>
<td>11-8</td>
<td>66</td>
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<td>6-8</td>
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*This patient received clinically ineffective transfusions for 7 cumulative days before the in vitro antibody discovery.
detectable in all cases but one. These patients will be referred to as "low responders."

Patients 11–20 (Table 1) have developed what is usually described as immune response to factor VIII. Patients 11–18 had a documented primary or anamnestic response. Patients 19 and 20 had not been transfused since 1971 and had a high and stable antibody titer and therefore were included in this group. Antibodies in these patients were discovered after an average of 22 cumulative exposure days (range 2–70 days) and maximum titers ranged from 3.0 to 1900 U/ml.

Patients' Responses to Stimulation With Factor VIII Antigen

Figure 1 is illustrative of the immune response of low responders following transfusions given over a period of several years; patient 6 had a quite stable antibody (0.25–0.5 U/ml) although stimulated repeatedly. After 10 mo without transfusions, his antibody became undetectable. Similar patterns were seen in patients 4, 5, 7, 8, and 9 (Table 1). The history of patient 1 has been previously reported.17 Six years after antibody detection, this patient was treated with large doses (up to 100 U/kg/day) of factor VIII for 3, 6, 8, and 3 days respectively, within a 5-mo period. His antibody titer did not rise above 3.6 U/ml in spite of intense stimulation. After the antibody had returned to an undetectable level, he received four transfusions at intervals of 3 mo; the antibody never exceeded

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Fig. 1. Immunization course of patient 6. Arrows indicate single injections of cryoprecipitate. Observed recovery in vivo (-----) is expressed in per cent of the expected recovery. The shaded area corresponds to antibody titers below the threshold of reliability. The antibody titer is indicated by open circles (o—o).
0.5 U/ml. Patient 2 had a definite increase in antibody titer on two occasions only over his last ten stimulations; patient 3 had a similar response pattern.

Antibody titers were evaluated in plasma samples drawn in patients 2-8 immediately before and 8-20 days after 16 single transfusions of cryoprecipitate. Before stimulation, the mean value was 0.38 ± 0.31 U/ml. At the anticipated peak of the anamnestic response, the mean value was 0.69 ± 0.45 U/ml. Pre- and postinfusion results were not significantly different (p ≤ 0.05).

In Vivo Factor VIII Recovery

Several factor VIII recovery studies were performed in all low responders. Results from five typical cases are shown in Table 2. Shortly or immediately before infusion, antibody titers ranged from 0.2 to 0.8 U/ml. The peak of factor VIII activity, observed 30-60 min postinfusion, was below the expected values. In most instances, the highest factor VIII plasma level was observed after 30 min, while, in the controls, the peak was more often found 60 min postinfusion.

In several cases, an abnormal in vivo recovery preceded the in vitro detection of an antibody or was contemporaneous with an antibody titer below 0.2 U/ml. Patient 10 illustrates this type of behavior (Fig. 2). This patient received 32 transfusions over a 2-yr period, with normal factor VIII recovery on two occasions. In June 1973, a questionable antibody (0.15 U/ml) was detected following a transfusion that resulted in a slightly abnormal recovery. Six months later, he received prophylactic treatment consisting of 20 ± 2 U/kg of factor VIII three times a week for 5 wk. During the last 2 wk of this treatment, the factor VIII recovery dropped progressively to 80%, 64%, and 23% of the expected values. Treatment was then stopped, and a low titer antibody appeared. After 4 mo without transfusions, replacement therapy was resumed and three successive factor VIII recoveries were 95%, 83%, and 80% of that expected.

Factor VIII recovery in vivo was plotted against antibody titer on an arith-
Fig. 2. Antibody occurrence in patient 10. Arrows indicate single injections of cryoprecipitate. Observed recovery in vivo (---) is expressed in per cent of the expected recovery. The shaded area corresponds to antibody titers below the threshold of reliability. The antibody titer is indicated by open circles (○).

metic scale utilizing data from 22 transfusions in seven cases (Fig. 3). Antibody titrations were performed on plasma samples collected immediately before the infusion of factor VIII. A significant correlation was found ($r = -0.65$). All recovery values but one were outside the normal range, even when antibodies were undetectable by in vitro techniques.

Factor VIII Survival

In most survival studies, factor VIII activity decreased rapidly between the first and the fourth hours postinfusion and then followed two different patterns (Table 2). One corresponded to a shortened half-disappearance time (patients 2b, 5b, 6a and c); the other to a normal one (patients 2a and c, 5a, 6b, and 7). Both types could be found in a given patient on different occasions.

Characterization of Antibodies to Factor VIII in Low Responders

Prolonged incubations of plasma samples from patients 2, 3, 6, and 8 with human, porcine, and bovine plasmas were performed over a 6-hr period at 37°C. A stable plateau of residual factor VIII developed only with human antigen. A pattern of progressive inactivation was observed with porcine antigen. In patients 2 and 8, residual human and porcine factor VIII were similar after 2 hr of incubation, expressing a high degree of cross-reactivity (Table 3). No inactivation of bovine factor VIII was ever found.
For technical reasons, dissociation studies were performed only in plasmas of patients 2 and 3 of the low responding group, 20 days after transfusion when their antibody titer was over 1 U/ml. The recovery of dissociated antibody was over 50% after 30 min of incubation at 56°C.

**DISCUSSION**

In hemophilia, the major obstacle to effective replacement therapy is the presence of antibodies which rapidly inactivate the procoagulant activity of the infused factor VIII. Early detection of these antibodies requires sensitive qualitative assays, and the treatment of patients is dependent on an accurate quantitative assay. Measurement of low titer antibodies raises problems and the ratio of antigen to antibody must be considered in order to maintain optimal conditions with the presence of a slight antigen excess. In this study, increasing volumes of antibody have been mixed with a constant volume of undiluted normal plasma. We have found this method to be reproducible in titrations of antibodies ranging from 0.2 to 1.0 U/ml, when the three antigen–antibody ratios are tested in the same experiment.

Analysis of the immunologic consequences of replacement therapy in hemophilia A showed that most hemophiliacs with an antibody had a stereotyped immune response following antigenic challenges. However, previous reports mentioned a few cases whose antibody titer remained low despite repeated stimulations with factor VIII. Analogous to the situations that have been observed in experimental immunology, we propose to designate these two types “high responders” and “low responders.”

| Table 3. Specificity of Antibodies From Low Responders |
|-------------------------------------------|---------------------------------------------|
| Residual Factor VIII (U/ml)               | Incubation Time With Human F. VIII (hr)     | Incubation Time With Porcine F. VIII (hr) |
| Patient                                 | 2   | 4   | 6       | 2   | 4   | 6       |
|                                          | 0.46 | 0.40 | 0.40    | 0.46 | 0.35 | 0.30    |
| 2                                       | 0.175 | 0.18 | 0.18    | 0.70 | 0.54 | 0.46    |
| 3                                       | 0.30 | 0.29 | 0.30    | 0.76 | 0.62 | 0.54    |
| 5                                       | 0.62 | 0.54 | 0.54    | 0.57 | 0.43 | 0.35    |

1 2 3 4 5 6
The low responders (Table 1, cases 1–10) were carefully studied in this report. Before antibody discovery, immunization schedules were similar to those observed in cases 11–18 and consisted predominantly of repeated single infusions of human cryoprecipitate. Factor VIII-containing blood products were also infused, and prolonged stimulation, required by surgical events, had taken place in both groups of patients. In the group of low responders, antibodies were discovered between the age of 6–14 yr, after an average of 48 cumulative exposure days to factor VIII (Table 1). Since all patients were at the Center at the time of antibody discovery, this figure was significantly higher than the 22 cumulative days observed in the group of high responders.

In addition to the large number of stimulations required to develop an antibody, low responders often did not react (patients 4–10) or reacted poorly (patients 1–3) to repeated antigenic stimulations. On 16 occasions, no significant rise was found when antibody titers were evaluated before and 8–20 days poststimulation. This expression of low immunologic reactivity contrasted with that observed in patients 11–20. In this group of high responders, renewed antigenic challenge after antibody discovery was always followed by a typical secondary response.

Since factor VIII recovery in vivo was systematically determined in newly referred patients who did not have antibodies demonstrable in vitro, abnormal recoveries were often the first sign of antibody occurrence. It is noteworthy that the peak of factor VIII was often observed 30 min postinfusion instead of 60 min, as observed in controls (Table 2). This fact, associated with factor VIII recovery below the expected value, was considered strongly suggestive of immunity to factor VIII. Since the test in vivo included the hazard of an anamnestic response, recovery studies were performed only when replacement therapy was required for treatment of bleeding episodes. The correlation between factor VIII recoveries and antibody titration performed immediately before transfusion (Fig. 3) indicated that the in vitro method indeed reflected the in vivo factor VIII neutralization.

In order to differentiate further high and low responders, some functional analyses of factor VIII antibodies were undertaken. Antibodies from low responders neutralized significant amounts of an heterologous antigen (porcine factor VIII) to which patients had not been sensitized (Table 3). This degree of cross-reactivity was larger than that observed with antibodies from high responders. Differences in antibody affinity between the two groups, as measurable by resistance to heat-induced dissociation of the factor VIII–anti-factor VIII complexes, were also found. In low responders the complexes dissociated at a fast rate and up to 80% of the antibody activity was released. When compared to high responders, the limited immune response of low responders might be related to a more limited repertoire of clones able to synthesize factor VIII antibody and/or an inability to select progressively clones producing antibodies with high affinity.

On the basis of data collected in this study, we suggest that hemophiliacs should be screened for the occurrence of antibody on two levels. (1) One should include both in vivo and in vitro detection of factor VIII neutralizing capacity; (2) one should attempt to discriminate between low and high responders. Only
data collected over a long period of time can meet the second requirement. The criteria for low responders would be occurrence of detectable in vivo and in vitro antibody to factor VIII, a return to normal in vivo recoveries weeks after the last stimulation, and inconstant and minimal secondary response after repeated transfusions.

It is difficult to give a valid estimate of the overall incidence of anti-factor VIII antibodies in hemophiliacs below 16 yr of age, since this type of patient is specifically referred to the Center for treatment. Among 30 hemophiliacs found immune to factor VIII during the period 1971–1975, 17 are high responders, 11 low responders, and follow-up remains insufficient to ascribe 2 patients to either group.

In clinical practice, the recognition of low responders is of importance because these patients can receive and benefit from replacement therapy without the limitations imposed by an overt immune response. In these patients, immunosuppressive therapy designed to avoid an anamnestic response is usually not justified.  

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REFERENCES


Antibodies to factor VIII. V. Patterns of immune response to factor VIII in hemophilia A

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