Immunoblot Analysis Shows Changes in Factor VIII Inhibitor Chain Specificity in Factor VIII Inhibitor Patients Over Time

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We have used immunoblotting of purified factor VIII (FVIII) to determine whether or not changes in FVIII chain specificity occur during the course of an inhibitor. Serial plasma samples from 15 inhibitor patients (13 hemophiliac and two spontaneous) were analyzed. Nine of the 15 antibodies, all with epitopes on the 44-kilodalton (Kd) thrombin fragment of the 92-Kd FVIII heavy chain and/or the 72-Kd thrombin fragment of the 80-Kd FVIII light chain, showed no change in FVIII chain specificity. However, six of the inhibitors analyzed showed changes in FVIII fragment specificity. Four inhibitors (three hemophiliac and one spontaneous) reactive with 72-Kd thrombin fragment also became reactive with the 44-Kd thrombin fragment after an anamnestic response to FVIII infusion. Another inhibitor with epitopes on both the 54-Kd and 44-Kd thrombin fragments lost most of its reactivity with the 44-Kd fragment but retained its reactivity with the 54-Kd fragment following a FVIII infusion. The inhibitor later regained its 44-Kd–fragment reactivity but lost its 54-Kd–fragment reactivity following treatment with FEIBA, FVIII inhibitor bypassing activity. The last inhibitor studied had an antibody to either the 44-Kd fragment or to both the 44-Kd and 72-Kd fragments during anamnestic responses to FVIII. These data indicate that a FVIII inhibitor patient can potentially produce antibody to multiple areas on the FVIII molecule and that this must be taken into account in the design of specific therapeutic products.

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MATERIALS AND METHODS

FVIII Preparation

FVIII was purified from commercial FVIII concentrate by immunoadsorbent chromatography as previously described.79 Thrombin digests were made by dialyzing purified FVIII against 0.02 mol/L imidazole, 0.15 mol/L sodium chloride, 0.002 mol/L calcium chloride, and 0.02% sodium azide, pH 7.0, overnight at 4°C. Purified human α-thrombin was then added to a final concentration of 10 U/mL, followed by incubation at 37°C for two hours. Thrombin was then inhibited by adding D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (Calbiochem-Behring Corp, La Jolla, CA) to a final concentration of 10 μmol/L.

Assays

FVIII inhibitor activity was measured in Bethesda units (BU) by the method of Kasper and coworkers.16

Electrophoresis

NaDodSO4 polyacrylamide gel electrophoresis of FVIII was done as follows: purified FVIII or thrombin-proteolyzed FVIII was dialyzed against sample buffer (0.01 mol/L sodium phosphate, 1% NaDodSO4, 10 mmol/L disodium EDTA, pH 7.0) overnight at room temperature. Additional NaDodSO4 was added to the dialyzed sample to bring the final concentration to 2%. No heating or reduction was performed. The FVIII was applied to 0.75-mm x 9-cm x 16-cm NaDodSO4, 7.5% polyacrylamide gels11 and subjected to electrophoresis in a Protean II electrophoresis apparatus (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer’s instructions. Bovine serum albumin (BSA) was used as a negative control protein, and either human IgG or affinity-purified goat antihuman IgG, IgA, and IgM14 were used for positive controls.

Immunoblotting

Electrophoretic transfer of FVIII from the gels to the nitrocellulose membranes was done by using a Hoeffer Transphor apparatus (Hoeffer Scientific Instruments, San Francisco) overnight at 4°C with the current set at 0.2 A. The transfer buffer contained 25 mmol/L Tris adjusted to pH 8.3 with glycine, 20% methanol, and 0.1% NaDodSO4. After transfer, the nitrocellulose membranes were processed as previously described except that the plasma samples were prepared for immunoblotting by heating at 60°C for 30 minutes and centrifuging briefly before use. Most autoradiographs represent a film exposure of 1 week. The inhibitor IgG was detected by using a monoclonal antibody to human IgG4 (or IgG1 or IgG3 where indicated) (ICN Immunobiologicals, Lisle, IL) as previously described.54

Patient Samples

The citrated plasma samples used in this study were collected from patients treated at the First Medical Clinic, University of Vienna, and were stored at –20°C. The samples were shipped on dry ice by air freight. Titters varied from 3 BU/mL to over 4,000 BU/mL.

RESULTS

Plasma samples from 15 FVIII inhibitor patients were taken at intervals during the course of their treatment. The time spans covered by the samples ranged from less than 1 month to 17 years. Thirteen of the inhibitors were from hemophiliacs, and two were from spontaneous inhibitors. All of the hemophilic inhibitors and one of the two spontaneous inhibitors showed anamnestic responses to infusion of human FVIII. Two of the hemophilic inhibitor patients were also treated with porcine FVIII, and two other hemophilic inhibitor patients were also treated with FEIBA, an activated clotting factor concentrate (ImmuNo, Vienna). Immunoblotting of all of these plasma samples by using purified human FVIII polypeptide fragments54 (Fig 1) showed that six of the 15 inhibitors had clear changes in FVIII polypeptide fragment reactivity during the course of the inhibitor.

Inhibitors With Changes in FVIII Fragment Specificity Over Time

Four of the six inhibitors that showed changes appeared to gain or lose reactivity with the 44-Kd thrombin fragment of the FVIII heavy chain (Fig 1) either completely or partially while maintaining light-chain reactivity during the course of the inhibitor. One of the two spontaneous inhibitors was included in this group. Conversely, a fifth inhibitor appeared to lose light-chain rather than heavy-chain reactivity over time. In addition, this patient had a strongly reactive and apparently nonneutralizing antibody to the middle section of the FVIII molecule that was present throughout. The sixth inhibitor had only heavy-chain specificity but showed differential reactivity toward its 54-Kd and 44-Kd thrombin fragments over time. These results are presented in Figs 2 through 7 and are described as follows.

Patient T.B. Figure 2A illustrates the course of this inhibitor, a high-titer antibody that showed anamnestic responses to intermittent doses of FVIII and FEIBA. Figure
2B shows the corresponding immunoblots from plasma sample dates A through D when using a monoclonal antibody specific for human IgG4.5,6 As seen in Fig 2A, in 1976 the patient had an anamnestic response to FVIII infusion, as shown by the increase in inhibitor titer. In 1977 an increase in titer was treated with FEIBA, which resulted in another anamnestic response, and immunoblots (Fig 2B, panels A and B) showed antibody directed against the 80-Kd FVIII light chain and its 72-Kd thrombin cleavage product. Later that year, FVIII was administered, and a major anamnestic response occurred (Fig 2A), with production of antibody to both the 80-Kd light and 92-Kd heavy chains of FVIII and their 72- and 44-Kd thrombin cleavage products as seen in Fig 2B, panel C. During 1978, multiple doses of FEIBA were administered (Fig 2B), and immunoblot analysis of a plasma sample (Fig 2B, panel D) showed persistence of the anti-light-chain antibody, with diminution of the anti-heavy-chain antibody. In 1980 (Fig 2A) continuous high-dose FVIII treatment was administered, and the antibody titer decreased to a low level. Thus, this inhibitor to the FVIII light chain showed gain and loss of FVIII heavy-chain reactivity over time.

Patient A.H. Figure 3A shows multiple anamnestic responses to FVIII infusion over a 12-year time span in a high-titer hemophilic patient. Immunoblots in Fig 3B show that two strong anamnestic responses to FVIII infusion in the sixth year (Fig 3A, 1978) involved antibodies to both the 92-Kd heavy and 80-Kd light chains of FVIII and their 44- and 72-Kd thrombin cleavage products (Fig 3B, panels B and D), while a weaker response in the tenth year (Fig 3A, 1982) resulted in the production of only anti-light-chain antibody (Fig 3B, panel I). As with the aforementioned inhibitor, gain and loss of FVIII heavy-chain reactivity and maintenance of FVIII light-chain reactivity was demonstrated.

Patient W.L. The immunoblots in Fig 4 show a similar
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Fig 3. (A) Course of inhibitor from patient A.H. (B) Autoradiographs of immunoblots from plasma sample dates A through I corresponding to Fig 3A. FVIII is in the left lane, and thrombin-treated FVIII is in the right lane. FVIII polypeptide mol wts are shown as kilodaltons. Arrows indicate changes in FVIII fragment specificity.

phenomenon in this hemophilic patient, who had very high titers following FVIII treatment. Panel A shows the presence of both anti-light-chain (80- and 72-Kd) and anti-heavy-chain (92- and 44-Kd) antibodies in 1972, which were associated with a titer of 1,280 BU/mL. However, a plasma sample taken 13 years later, which had a higher titer of 3,636 BU/mL (panel B), showed a predominantly anti-light-chain antibody response. These data suggest that the presence of antibody to both the FVIII heavy chain and light chain does not necessarily indicate a maximum inhibitory titer. It is likely that the quantity and heterogeneity of inhibitor antibodies are also factors.

Patient H.A. Figure 5 illustrates a spontaneous inhibitor from a female patient who had systemic lupus. Her antibody also changed in FVIII (92- and 44-kD) heavy chain and (80- and 72-Kd) light chain specificity over time. Figure 5A shows the course of the inhibitor, which was treated with immunosuppressive drugs as indicated. FVIII administered in September 1977 caused a rise in inhibitor titer, which fluctuated above 100 BU/mL for over a year. A decrease in titer was observed during cyclophosphamide treatment in the first half of 1979. Figure 5B shows the corresponding immunoblots. Panels A and B show that the inhibitor was initially directed against only the FVIII light chain. At the end of the second year (panel C), however, a weakly reactive antibody to the heavy chain appeared and became strongly reactive 1.5 months later (panel D) despite preceding doses of cyclophosphamide (Fig 5A). Panel E in Fig 5B shows a loss of heavy-chain reactivity but retention of anti-light-chain antibody as the inhibitor titer decreased during continuous cyclophosphamide treatment. A plasma sample from March
1980 showed a weak anti-light-chain antibody and a low titer (Fig 5B, panel F). Again, the presence of antibody to both the FVIII heavy and light chains as compared with antibody to only the FVIII light chain was not indicative of a maximal titer and appeared despite immunosuppressant therapy.

**Patient G.R.** Figure 6 (A and B) shows interesting data from a hemophiliac with a high-titer antibody and typical anamnestic responses. A plasma sample taken in 1979 (panel A in Fig 6B) showed antibody to both the 92-Kd (and its 44-Kd thrombin fragment) FVIII heavy chain and the 80-Kd (and its 72-Kd thrombin fragment) FVIII light chain as well as a very strongly reactive antibody to the middle section (Fig 1) of the FVIII molecule (seen as heavy, dark material above 92 Kd in panels A through D for FVIII and diffuse, faster-migrating material after thrombin treatment). Human antibody to the middle section of FVIII has been previously described by us and appears to be either noninhibitory or associated with a very low titer. Figure 6A shows the effect of continuous high-dose FVIII treatment over a 9-month period with corresponding immunoblots in Fig 6B, panels B through D. A sample taken in October 1982 (Fig 6B, panel B) shows the presence of only the anti-middle section antibody and a titer of 3 BU/mL (a qualitatively similar immunoblot of a plasma sample taken before that in panel A had no measurable inhibitory titer). Later that month, an anamnestic rise in titer occurred after FVIII infusion, and panel C reveals the presence of only anti-heavy-chain antibody, with the continued presence of antibody to the middle section. In April 1983, a mild rise in titer occurred, again associated with anti-heavy-chain antibody (panel D), although it was very weakly reactive.

Thus, this patient provides a striking example of the occurrence of an antibody with little or no neutralizing titer that is directed against a portion of FVIII that is not critical to procoagulant function. In addition, the production of antibody to the FVIII light chain in 1979 did not recur with the anamnestic response of 1982 as compared with patients T.B. and A.H., whose inhibitors lost FVIII heavy-chain rather than light-chain reactivity over time.

**Patient H.W.** Figure 7 shows data for another hemophiliac inhibitor that showed changes in FVIII epitope specificity. However, unlike the aforementioned patients, this patient had antibody only to the 92-Kd FVIII heavy chain.
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throughout the 17-year time period in which he was followed. The changes involved differential reactivity of the 54- and 44-Kd thrombin fragments of the FVIII heavy chain. Figure 7A shows multiple, high, anamnestic responses to FVIII infusion during the first 10 years and a milder anamnestic response to FEIBA during the 11th year (1978) followed by a slow decrease in titer. In Fig 7B, panel A shows that during the first year an anamnestic response with antibody to the 92-Kd FVIII heavy chain involved both the 54- and 44-Kd thrombin fragments. This is only the second inhibitor plasma out of 76 that we have screened that had strong reactivity with the 54-Kd thrombin fragment. The majority of inhibitors were reactive with only the 44-Kd fragment. A decline in titer at the end of the seventh year (Fig 7A) correlated with a weakly reactive antibody that was predominantly to the 44-Kd thrombin fragment (Fig 7B, panel B). However, panel C shows that at the beginning of the eighth year an anamnestic response to FVIII infusion resulted in antibody mainly to the 54-Kd thrombin fragment. In the 11th year, very weak antibody reactivity and a low titer (panel D) was followed by an anamnestic response to FEIBA in which the predominant antibody was to the 44-Kd thrombin fragment (panel E), with only weak 54-kD fragment reactivity. Thus, panels A, C, and E of Fig 7B clearly show changes in the degree of inhibitor reactivity with the two thrombin fragments of the FVIII heavy chain. Two samples taken in the 12th and 16th years during the decline in titer following FEIBA administration showed weak reactivity with the 44-Kd fragment (Fig 7B, panels F and G). In addition, immunoblotting of the samples in panels A, C, and E of Fig 7B with monoclonal antibodies to IgG1 or IgG3 rather than IgG4 showed that IgG1 was present only in the sample in panel C and had similar fragment specificity (not shown). Taken together, these data suggest the predominance of different antibody-producing clones over time.

Inhibitors With No Change in FVIII Fragment Specificity Over Time

Of the nine inhibitors that showed no change in FVIII fragment reactivity over the time periods studied, three reacted only with the 44-Kd thrombin fragment of the 92-Kd FVIII heavy chain (the other spontaneous inhibitor was in this group), two reacted only with the FVIII light chain, and four showed reactivity with both the heavy and light chains of FVIII throughout. Data for two of these inhibitors are shown in Figs 8 and 9.

Patient W.S. The patient in Fig 8 is a hemophiliac with a high-titer inhibitor who was treated with porcine FVIII and had a very high anamnestic response. The cross-reactivity between human and porcine FVIII was 10% before and almost 100% afterward. Interestingly, the immunoblots of plasma samples taken during this 1-month period showed only anti-heavy-chain antibody. When monoclonal antibodies to IgG1 or IgG3 were used instead of anti-IgG4 in panels A through D of Fig 8, only panels C and D were weakly positive for IgG1, and IgG3 was not detected in any samples. Thus, porcine FVIII treatment did not immediately result in

Fig 6. (A) Course of inhibitor from patient G.R. (B) Autoradiographs of immunoblots from plasma sample dates A through D. B through D correspond to Fig 6A. FVIII is in the left lane, and thrombin-treated FVIII is in the right lane. FVIII polypeptide mol wts are shown as kilodaltons. Arrows indicate changes in FVIII fragment specificity.

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new FVIII fragment reactivities or a strong IgG1 response. Nevertheless, an increase in the number of heavy-chain epitopes could have occurred and would not have been detected here.

**Patient G.K.** Figure 9 presents data for a spontaneous inhibitor with no change in FVIII fragment specificity over time. This female patient developed the inhibitor without having any underlying disease. Surprisingly, she had an anamnestic responses to treatment with FVIII (Fig 9A). Her antibody titer eventually declined during immunosuppressive therapy over the 3.5-year period shown (1972 to 1975). Immunoblots in Fig 9B, panels A through H, show an antibody to the 44-Kd thrombin fragment of the 92-Kd FVIII heavy chain, with no changes other than weakened reactivity with decreasing titer.

**Patients R.M., P.B., F.T., J.H., M.G., H.H., and R.K.** Seven other inhibitor patient plasma samples were analyzed over periods ranging from 0.5 months to 9.5 years as follows: R.M., 9.5 years; P.B., 8.6 years; F.T., 7.1 years; J.H., 2.3 years; M.G., 4.3 years; H.H., 5 years; and R.K., 0.5 month. No changes in FVIII chain specificity could be detected in any of these patients by immunoblotting (data not shown), although plasma samples from some intermediate years were not available and could have shown changes. They included inhibitors specific for the FVIII light chain, the FVIII heavy chain, or both of these chains: patient R.M.
was a hemophiliac with a high-titer antibody; P.B. was a hemophiliac with a high-titer antibody who had been treated with high-dose FVIII until his antibody disappeared, and he subsequently had no further anamnestic response to FVIII treatment; patients F.T. and J.H. were hemophiliacs with high-titer antibodies; patient M.G. was the only patient analyzed who had moderate rather than severe hemophilia A (his FVIII level was 2%); patient H.H. was a hemophiliac with a high-titer antibody; and patient R.K. was also a hemophiliac who first developed an inhibitor following surgery and was treated with porcine FVIII. Thus, these seven hemophilia A patients with FVIII inhibitors of varying type and titer did not appear to change FVIII fragment specificity over time.

DISCUSSION

The finding that six of 15 FVIII inhibitors studied by immunoblotting showed changes in FVIII fragment specificity over time probably represents a minimum estimate of the frequency of this phenomenon. The data show that changes can occur within a few months and could be missed in plasma samples analyzed at infrequent intervals. The data also demonstrate the heterogeneity of inhibitors since the changes in FVIII fragment reactivities indicate the appearance and disappearance, or predominance, of different immunoglobulin-producing clones over time. Both hemophilic and spontaneous inhibitors showed changes. From these data, there does not appear to be any basis on which to predict whether an inhibitor patient will show a change in inhibitor specificity or when it will occur.

In addition, the two patients (H.W. and W.S.) analyzed for the presence of inhibitor IgG1 and IgG3 in addition to IgG4 were found to contain IgG1. The IgG1 was present in only one of the H.W. samples and had the same FVIII fragment reactivity as did the IgG4. In the case of W.S., IgG1 appeared only weakly in his high-titer samples after an anamnestic response to porcine FVIII. These data again

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Fig 8. Autoradiographs of immunoblots from plasma sample dates A through D for patient W.S. FVIII is in the left lane, and thrombin-treated FVIII is in the right lane. FVIII polypeptide mol wts are shown as kilodaltons.

Fig 9. (A) Course of inhibitor from patient G.K. AZA, azathioprine. (B) Autoradiographs of immunoblots from plasma sample dates A through H corresponding to Fig 9A. FVIII is in the left lane, and thrombin-treated FVIII is in the right lane. FVIII polypeptide mol wts are shown as kilodaltons.
indicate that the inhibitor antibody response is restricted and that changes in FVIII fragment reactivities were not IgG subclass dependent.

The variables present in these cases included treatment with FVIII, porcine FVIII, FEIBA, and immunosuppressants. In the case of patients T.B. and H.W., who had anamnestic response to FEIBA, the possibility exists that proteolytic fragments of FVIII present in the FEIBA could have specifically stimulated these responses. In the case of T.B., the antibody was to the FVIII light chain, whereas in the case of H.W., it was to the FVIII heavy chain, which suggests that both are present in FEIBA. In the case of patients W.S. and R.K., who were treated with porcine FVIII, a reasonable prediction is that antibody would be produced to many FVIII epitopes, although this was not the case. However, these two patients were followed for only 1 and 0.5 months, respectively, and a longer time period may be required for a major polyclonal response to heterologous FVIII. In patient H.A., a change in FVIII fragment specificity occurred despite continuous immunosuppressive therapy, which suggests that the immune response to FVIII may be controlled in a very specific manner. Another aspect of these data is that many of the samples studied were collected before 1980 and represent immunologic responses in inhibitor patients before the spread of the human immunodeficiency virus. This information is particularly valuable due to the obvious potential of this virus to create aberrations in the inhibitor response and complicate the interpretation of current studies.

These findings have particular significance in the therapy for FVIII inhibitors. Recombinant DNA technology offers the possibility of producing specific inhibitor-neutralizing fragments of FVIII, and detailed characterization of FVIII inhibitor epitopes as well as knowledge of the heterogeneity of the inhibitor response will allow the design of such therapeutic products. From these data it is clear that a FVIII inhibitor patient can potentially produce neutralizing antibody to both the heavy and light chains of FVIII at any time and that further characterization of all these epitopes is critical to the design of specific therapeutic products.

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