Effects of Plasmin on Human Factor VIII (AHF)

By R. Pasquini and E. J. Hershgold

Highly purified, fibrinogen-free human factor VIII was incubated with plasmin, and the liberated split products of the factor VIII were analyzed by gel filtration, acrylamide gel electrophoresis, bioassay, and for immunologic reactivity. At least three fragments retaining different antigenic determinants are released from the factor VIII after prolonged digestion and at least three new fragments are seen in acrylamide gel electrophoresis. The split products were not anticoagulant in the factor VIII activity assay. In fact, the breakdown products in the hydrolysate increased the factor VIII activity of normal plasma mixed with it. Therefore, it is not likely that the factor VIII split products formed in fibrinolytic states contribute actively to the hemorrhagic diathesis.

The reported effects of plasmin on factor VIII have been limited to measurement of the loss of the factor VIII activity. Studies of the effects of plasmin on fibrinogen have progressed further and have shown not only that the split products liberated from fibrinogen act as anticoagulants, but also have revealed details of the antigenic structure of fibrinogen.

Since we have available a highly purified, fibrinogen-free factor VIII and a specific rabbit antiserum to it, similar studies of the effects of plasmin on factor VIII appeared profitable. In the present study, we have examined the possible anticoagulant effects of plasmin-produced factor VIII split products and their immunologic reactivity.

Materials and Methods

Plasmin was provided by Cutter Laboratories as a urokinase-activated plasminogen containing 2.4 casein units/mg protein (Homolysin). No other enzymatic activities are known for this product, and it shows only one band in acrylamide gel electrophoresis.

Highly purified factor VIII was prepared using the method of Hershgold et al. The final product was fibrinogen free, as revealed by immunodiffusion methods, and contained 0.7 mg/ml protein. No more than 8% of this was contaminants, of which only aggregated IgG globulin and IgM globulin could be identified. This factor VIII solution is 0.2 M in e-aminocaproic acid (EACA; Sigma Chemicals, St. Louis, Mo.). To reduce the content of EACA to insignificant levels, 3 ml of the preparation were dialyzed for 3 hr against 1 liter of buffer at room temperature, 0.05 M of Tris in 0.1 M NaCl, pH 7.8. The buffer was changed every hour.

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Reaction of Factor VIII With Plasmin and Separation of Reaction Products

The dialyzed factor VIII was incubated with plasmin, 10 casein units/ml of factor VIII, for 1 hr at 37°C. Three-milliter samples of this plasmin-factor VIII mixture were chromatographed on 2.5% agarose gel granules (prepared in our laboratory as previously described) in a 2.3 × 35 cm column, as were dialyzed but untreated factor VIII and plasmin alone. Flow rates were maintained at 6-7 ml/sq cm/hr; 4-ml fractions were collected. The columns were equilibrated and eluted with the Tris-NaCl buffer and were operated at room temperature.

Ultraviolet absorption at 280 nm was used to determine the protein elution pattern. The chromatographed fractions were concentrated 30-fold by means of Diaflo ultrafiltration cells (Amicon, Lexington, Mass.) using UM-30 or UM-10 membranes as appropriate. Protein was estimated by the Lowry method.

Assay for Factor VIII Activity

The one-stage method was used to determine activity in all fractions. This assay was a modification of the method of Rapaport et al., utilizing activated cephaloplastin (Dade).

Effects of Plasmin Treatment of Factor VIII on the Factor VIII Activity

The fractions isolated as above by chromatography of plasmin-treated factor VIII both before and after concentration, as well as dialyzed factor VIII alone, plasmin alone, and the original factor VIII-plasmin incubation mixture, were each incubated with equal parts of a normal plasma pool for 1 hr at room temperature. Factor VIII assays were then performed on these mixtures. In addition, a factor VIII-plasmin mixture was incubated at 37°C, and the reaction was stopped at 1, 3, 5, 10, 15, 30, and 60 min by adding EACA to give a final concentration of 0.2 M. These mixtures were then incubated with equal parts of normal plasma and were also assayed for factor VIII activity to assess the possible anticoagulant effect of the fractions.

Immunoelectrophoretic and Immunodiffusion Analyses of Factor VIII Split Products

The fractions collected from the agarose gel filtration of the plasmin-factor VIII mixtures, and of the factor VIII preparation alone, were analyzed by immunoelectrophoresis and immunodiffusion methods. The immunodiffusion method and the production of rabbit antiserum to human factor VIII have been described previously. This antiserum, prior to absorption treatment, has been shown by Stites et al. to react not at all or only weakly with plasma from patients with von Willebrand's disease in a hemagglutination system. To render the antiserum specific for factor VIII it was absorbed with von Willebrand's plasma; 3 volumes of von Willebrand's plasma were mixed with 7 volumes of antiserum, and this mixture was incubated at 37°C for 1 hr and then allowed to stand at 4°C overnight. The precipitate that formed was removed by centrifugation. The absorbed antiserum gave a single line with our factor VIII preparation in immunodiffusion and immunoelectrophoresis, and a 1:50 dilution in normal plasma inhibited at least 50% of the factor VIII activity of the plasma.

Factor VIII Hemagglutination-inhibition Titer

Hemagglutination-inhibition titers were performed, utilizing O+ human red blood cells coated with the highly purified factor VIII and the method described by Merskey et al.

Polyacrylamide Gel Electrophoresis

Seven and one-half per cent polyacrylamide gel slab electrophoresis was performed by the method described by E.C. Apparatus Corp., Philadelphia, Pa. Gels were stained with Coomassie blue. A Tris-EDTA-borate buffer, pH 8.4, was used with 300 V applied for 2 hr.
RESULTS

Biological Activity

Highly purified factor VIII (0.7 mg/ml) when exposed to plasmin lost all of its biological activity within the first minute of exposure. In addition, none of the fractions obtained from chromatographed, hour-incubated plasmin-factor VIII mixtures revealed measurable biological activity, even after their 30-fold concentration in an attempt to bring out trace amounts of activity.

Hydrolysate Analysis

The plasmin-factor VIII mixture, when analyzed by agarose gel filtration, consistently revealed the elution pattern observed in Fig. 1. Two main protein peaks were noted. The elution volume of the first peak (F₁) was similar to that of the untreated factor VIII. Although considerably lower OD at 280 nm was consistently obtained, the OD of this peak is still significant. Protein assay revealed it to contain only 0.19 mg/ml of protein after concentration. These findings are consistent with our previous demonstration of a high, light-scattering effect associated with factor VIII.³ This peak may then represent undegraded or slightly degraded material or possibly factor VIII from which the majority of protein has been split off, leaving aggregates of other constituents of the molecule that still absorb at 280 nm.

The second peak (F₂) eluted quite far from the first peak. This peak was broader and showed a higher absorbance at 280 nm when compared with plasmin alone. After concentration, this fraction contained 0.61 mg/ml of protein.

The content of fractions F₁ and F₂ was investigated by electrophoresis in 7.5% polyacrylamide gel (Fig. 2). The F₁ fraction behaved similarly to untreated factor VIII, i.e., it did not enter the gel. The F₂ fraction contained several fragments of which at least three were distinct from plasmin alone. There is also present a dark band just at the gel top, which may be a single species or consist of yet unseparated or denatured material.

Immunologic Studies

The concentrated fractions F₁ and F₂ were examined by immunodiffusion and immunoelectrophoresis for factor VIII antigenic reactivity. In these studies, fraction F₁ occasionally showed a very weak line of precipitation with antifactor VIII rabbit antibody (not demonstrated here). On some occasions, no line at all was obtained with these samples that contained very little protein. Fraction F₂, when analyzed by immunodiffusion, revealed two distinct lines of precipitation (Fig. 3).

When the factor VIII antiserum was absorbed with fraction F₂, immunodiffusion did not reveal precipitation lines with the F₂ fraction, but the absorbed antiserum was still able to show a precipitin line with untreated factor VIII (Fig. 4).

In multiple experiments, immunoelectrophoresis revealed two lines of precipitation displaced from antigen well toward the anode. One of these bands does not reproduce sharply and is indicated by the arrow (Fig. 5).
ml concentrated to give fraction F₂, from chromatography of the factor VIII-plasmin mixture. Volume collected between 100-130 ml gave too small amounts of protein for study.

The hemagglutination-inhibition activity of the fragments in fraction F₂ was higher (i.e., more antibody-combining power), titer 1/128, than that obtained with Fraction F₁, titer 1/32. The titer of the 30-fold concentrated eluate peak of untreated factor VIII was 1/256 (Table 1).

**Effects of Factor VIII Split Products on the Factor VIII Assay**

To assess the effects of factor VIII split products on the factor VIII assay, both the original factor VIII-plasmin incubation mixture and the fractions isolated from it by gel filtration were incubated for 1 hr at room temperature with equal parts from a normal plasma pool (16 donors). An anticoagulant effect was not detected in the factor VIII activity assay. When the normal plasma pool was incubated with the fractions collected late in the F₅ effluent, i.e., those containing the smaller fragments, a consistent increase of factor VIII activity was found (Fig. 6).

**DISCUSSION**

We have confirmed the findings reported by others¹ that plasmin rapidly destroys the biological activity of factor VIII. Associated with this loss of
activity after 1-hr digestion with plasmin, three new fragments were detected in polyacrylamide gel electrophoresis (Fig. 2). It seems likely that these fragments are the major final products of the plasmin-factor VIII interaction, since large amounts of plasmin were used in a prolonged incubation. We have restricted this study to analysis of the late stages of digestion products. Additional investigation of sequential changes with time of incubation might be expected to show evolution of intermediate products. The whole question of the molecular determinants of factor VIII activity is largely open, and knowledge of those products that are split off by plasmin will require considerably more analysis.

From the immunodiffusion (Fig. 3) and immunoelectrophoretic studies (Fig. 5), it appears that two fragments each retain different factor VIII antigenic determinants. It is also probable that at least one more set of determinants is present on the parent molecule, since when the antibody was absorbed with the F3 fragments, the absorbed antiserum was still able to react with untreated factor VIII, although no precipitin lines were seen with the F3 fraction (Fig. 4).
Table 1. Comparison of Hemagglutination-Inhibition Titers of Various Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Titer</th>
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</thead>
<tbody>
<tr>
<td>F₁, plasmin-treated factor VIII</td>
<td>1/32</td>
</tr>
<tr>
<td>F₂, plasmin-treated factor VIII</td>
<td>1/128</td>
</tr>
<tr>
<td>From single peak obtained after chromatography of untreated factor VIII</td>
<td>1/256</td>
</tr>
<tr>
<td>Plasmin (30 casein U/ml)</td>
<td>0</td>
</tr>
<tr>
<td>Normal plasma pool (16 donors)</td>
<td>1/16</td>
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</tbody>
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Since at least one or more antigenic determinants must be present on the whole untreated molecule to react with the absorbed antibody, and these were not consistently located in the analyses of the split products, it is possible that they may have been destroyed during the enzymatic treatment. The additional antigens, however, may also be represented by the F₁ fraction and be inconsistently revealed with the methods used.

The finding of a much higher hemagglutination-inhibition titer in the F₂ fragments suggests that the majority of the factor VIII antigenic determinants are released by plasmin and appear in these fragments. Moreover, the less likely possibility of exposure of new binding sites should be considered, since this might also increase the hemagglutination-inhibition titer. However, protein analyses showed that most of the protein was found in the second peak; even after concentration of the first peak very low amounts of protein were recovered.

The fragments released by plasmin, regardless of the length of time of hydrolysis, did not interfere with the factor VIII assay. The effects of the factor VIII degradation products in other specific stages of the intrinsic coagulation system were not studied. If such effects were present, it would be likely that an anticoagulant effect on the assay system used would be observed.

In four consecutive experiments, an increase of factor VIII activity of normal plasma was found after it was incubated with the smaller (F₂) fragments eluted from agarose gel filtration. The explanation for this phenomenon is not clear. Whether this finding is determined by the factor VIII fragments or a plasmin-factor VIII fragment complex acting to activate one or more

Fig. 5. Immunoelectrophoresis employing rabbit antiserum to human factor VIII. Upper, F₂ with arrow indicating faint line that does not reproduce well; lower, factor VIII concentrate.
EFFECTS OF PLASMIN ON HUMAN FACTOR VIII

Fig. 6. This group superimposes two steps in analysis. Solid line indicates the effluent distribution of products of factor VIII-plasmin hydrolysis passed through a 21/2 agarose gel column. Dotted line represents factor VIII activity obtained when equal volumes of normal plasma are mixed with the column eluate at congruent points.

coagulation factors was not determined. It is possible that this effect is etiologically related to the increase of factor VIII activity measured by the one-stage technique that may be seen in fibrinolytic states.8

Since the factor VIII fragments produced by plasmin were not anticoagulant in these studies, it seems unlikely that the factor VIII fragments liberated by plasmin in a fibrinolytic state will contribute to the hemorrhagic diathesis. This is in contrast to the known anticoagulant activity of fibrin split products.1

REFERENCES

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