Mechanism of Factor VIII Inactivation by Human Antibodies. III. Proteolytic Cleavage of Factor VIII:C and C Antigen by Thrombin*

JOHN LAZARCHICK, M.D., MARK A. ASHBY, M.D., JOHN J. LAZARCHICK, B.A., and DONALD A. SENS, Ph.D.

Departments of Laboratory Medicine and Pathology, Medical University of South Carolina, Charleston, SC 29425

ABSTRACT

Thrombin activation of factor VIII results in a marked, but transient, increase in factor VIII procoagulant activity. This proteolytic process has been examined by immobilizing factor VIII in a solid phase system using mouse monoclonal antibody specific for the factor VIII related antigen. These studies demonstrate that thrombin activation is the result of proteolytic cleavage from the factor VIII:C protein of a peptide fragment which contains both the factor VIII:C procoagulant and VIII:C antigen sites recognized by human antibodies.

Introduction

Significant advances have been made recently in the area of hemophilia A research, particularly with the cloning and isolation of the factors VIII:C gene and the anticipation of large scale production of purified factor VIII:C being made available for therapeutic use.9 On a biochemical level, a number of investigators have demonstrated that activation of factor VIII by thrombin results in the formation of low molecular weight fragments which can be recognized using protein electrophoretic procedures by immunoreactivity with mouse monoclonal antibodies to human factor VIII:C.3,8 Since these latter antibodies are reactive with distinct regions on the VIII:C molecule, it is impossible to determine the relationship of these findings to the area on the VIII:C protein recognized by human antifactor antibody which results in inhibition of VIII:C procoagulant activity.

This study was undertaken to examine thrombin activation of factor VIII which was selectively immobilized using mouse monoclonal anti-human factor VIII antibody specific for the factor VIII related protein. Under these conditions, proteolytic activation by thrombin results in the simultaneous liberation of factor VIII:C procoagulant and factor VIII:C antigen activities and suggests the peptide sites involved with these functions are iden-
tical or at least contained on the same proteolytic fragment.

Materials and Methods

Factor VIII sources in this study included pooled normal plasma prepared as previously described and partially purified factor VIII material obtained from gel chromatography on Biorad A 1.5 M of a lyophilized factor VIII concentrate. Borate buffered saline was the elution buffer for the preparation.

Factor VIII procoagulant activity was measured in a one-step assay utilizing a factor VIII deficient plasma as substrate. Factor VIII:C antigen and R antigen assays were performed using immunoradiometric assay system as previously described. All factor VIII assay results for factor VIII C, C antigen, and R antigen were based on their value relative to that of pooled normal plasma (100 U per dl).

Mouse monoclonal antibody was prepared utilizing classical hybridoma techniques. In brief, the mice were immunized at scheduled intervals over a six week period with purified human factor VIII. Spleens were then surgically removed and splenic cells teased from the mashed organ. These cells were fused with clone X63-Ag 8.653 myeloma cells using 50 percent polyethylene glycol. The fused cells were then plated in limiting dilutions into tissue culture wells and placed in a tissue culture incubator. Well supernatants were then assayed at variable times for the presence of antifactor VIII:R antigen reactivity. Positive wells were replated in limiting dilution and the culture process repeated. Positive wells which had grown to confluency were then transferred to culture flasks to expand antibody productivity. Spent media from the flasks were pooled and served as the antibody source. In addition, monoclonal antibody material was also obtained following intraperitoneal injection of positive cultures into mice with subsequent ascites formation.

Monoclonal antibody culture supernatants and ascitic fluid were precipitated with 40 percent saturated ammonium sulfate, pH 8.0. The precipitate was dissolved with distilled water and extensively dialyzed against borate buffered saline. The protein concentrate was covalently bound to Sepharose 2B-CL beads by the cyanogen bromide method. Control beads were prepared in an identical manner, except that a protein concentrate which did not contain factor VIII antibody was covalently bound in place of the monoclonal antibody material.

Prior to each experiment, an aliquot of the immune and non-immune beads were washed with borate buffered saline (BBS)/0.1 percent bovine serum albumin (BSA). After centrifugation and removal of the supernatant, the beads were then incubated with 0.4 ml of the factor VIII source for 30 minutes at room temperature with constant stirring. The mixture was then centrifuged and the supernatant removed. This was followed by washing the beads with one ml aliquots of BBS five times. The supernatant was saved and later assayed for factor VIII activities. After these extensive washing cycles, no residual factor VIII parameters were measurable in the supernatants.

An 0.5 ml aliquot of either human (0.025 μg per ml) or bovine (0.04 μg per ml) thrombin was then added to each set of beads. This volume of thrombin solution was used to permit multiple-time samples to be obtained. Aliquots were removed from the mixture at 30 seconds, and then 3, 6, 9, and 12 minute intervals and assessed for factor VIII:C. All procoagulant assays were performed at a

* Gift from Ken Schroer.
1/20 dilution in borate buffered saline. The same aliquots were also assayed for VIIIC antigen and VIIIIR antigen either the same day or were frozen at −70°C for later analysis. The thrombin concentration after dilution to 1/20 in borate buffered saline gave a clotting time similar to the borate buffered saline control. Additional controls were performed in which factor VIII deficient plasma obtained from a patient with severe hemophilia A (factor VIIIC <1 U per dl) were incubated with beads instead of the pooled normal plasma. The specificity of procoagulant activity generated after the thrombin exposure of the test beads was also determined using factor IX deficient human plasma as substrate.

Results

The three different processed ascitic fluid and tissue culture preparations containing the monoclonal antihuman factor VIII R antigen antibodies were used in these studies. In addition, no cross-reactivity with any other human serum protein was evident. None of these preparations had any antifactor VIII procoagulant activity. The exact site of antigenic reactivity on the factor VIII related protein was not examined further. The binding efficiency for coupling of these monoclonal antibody materials to the sepharose beads ranged from 73 to 92 percent. Control beads preparations gave similar binding efficiency results.

For the initial experiments, aliquots of packed immunized beads ranging from 0.1 to 0.5 ml were incubated with 0.5 ml of pooled normal plasma to determine first if the beads would selectively bind factor VIII and to determine the maximally efficient ratio. Based on the recovery of factor VIIIC in the supernatant fluid, it was elected to use a 1:1 ratio of bead volume to pooled normal plasma volume for all subsequent experiments. Similar experiments were performed with the partially purified factor VIII preparation, however, because of the relative amounts of factor VIIIIR antigen in this material which were so much greater than the factor VIIIC. The amount of intact factor VIII bound/bead volume was less than when pooled normal plasma was used as the factor VIII source.

After each set of immune and non-immune beads was incubated for 30 minutes with the pooled normal plasma, the mixtures were centrifuged and supernatant removed. The beads then were washed with a one ml aliquot of borate buffered saline. This step was repeated five times. After each subsequent centrifugation, each supernatant was assayed for factor VIII parameters. This step essentially removed any unbound factor VIII from the mixtures.

For the thrombin activation step, an 0.5 ml aliquot of 0.025 ml of human thrombin or 0.04 U per ml of bovine thrombin was added to each bead preparation in the manner so as not to disturb the bead pellet. The supernatant was then assayed for factor VIII procoagulant activity by removing 50 ml aliquots at timed intervals and immediately making a 1:20 dilution of this aliquot in BBS. The mixtures were briefly centrifuged prior to removal of these aliquots to ensure that beads to which factor VIII was bound were not carried over into the assay system. The typical pattern of factor VIII activation is shown in table I. Within 30 seconds after the addition of thrombin, VIIIC activity was evident in the supernatant in the immunized bead but not the control bead preparation. Peak activity was evident at the three minute point, followed over the next nine minutes by gradual loss of procoagulant activity. No factor VIIIC was evident at any time in the control bead
THROMBIN ACTIVATION OF FACTOR VIII

TABLE I
Thrombin Activation of Immunoabsorbed Factor VIII

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>Immune Beads</th>
<th>Control Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>9.2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>18.6</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>6.3</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Factor VIII:C - procoagulant activity

Activation was performed with 0.025 U/ml of human thrombin. Aliquots from the supernatant were assayed at a 1:20 dilution in borate buffered saline.

preparation, eliminating the possibility that the procoagulant activity generated in the supernatant might be due to the presence of thrombin activity carried over into the assay system.

Two additional controls were performed to ensure that the results seen were due to an effect of thrombin on bound factor VIII. When plasma from a patient with severe hemophilia A was used as the factor VIII source and incubated with immune beads, no procoagulant activity could be demonstrated after thrombin activation of the beads. In another experiment, factor IX deficient plasma was used as the substrate in the indicator assay. Again, no shortening of the clotting time could be demonstrated in the supernatant after the addition of thrombin. Both of these results indicate that the procoagulant activity present in the supernatant from the immune beads was due to the release of factor VIII:C from the immune beads.

Although these data are compatible with thrombin causing a release of factor VIII:C activity from the immunized beads, it did not distinguish whether this was due to release of intact factor VIII containing factor VIII related antigen, to the release of factor VIII:C with associated C antigen, or to the release of factor VIII:C unassociated with C antigen. The limited volume of reactants using these previous experiments precluded us from measuring all factor VIII parameters and necessitated expanding the reacting volumes. The relative ratios of beads to normal pool plasma to thrombin solution, however, were kept the same. The results with this modification are shown in table II. It is evident that no measurable factor VIII:R antigen was released from the beads during thrombin activation. Factor VIII:C is readily detectable with peak activity again evident at the three minute point. In addition, factor VIII:C antigen is also present in the supernatant, although the concentrations are far less than the corresponding VIII:C values at each assay point. This discordance in factor VIII:C/C antigen was evident on all subsequent studies performed. These results are most consistent with thrombin activation of factor VIII being the result of proteolytic clip in the factor VIII procoagulant molecule resulting in the cleavage of an activated protein fragment(s) which retains the antigenic site recognized by human anti-factor antibodies.

Discussion

Monoclonal antibodies used in this study were of high titer and were reac-

TABLE II
Factor VIII Parameters Proteolytically Cleaved by Thrombin Activation of Factor VIII

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>Factor VIII* (U/dl)</th>
<th>C:Ag</th>
<th>R:Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>5.3</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>20.8</td>
<td>9.9</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>8.4</td>
<td>6.6</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>1.5</td>
<td>5.8</td>
<td>0</td>
</tr>
</tbody>
</table>

*C = procoagulant activity; C:Ag = procoagulant antigen; R:Ag = related protein antigen.

The immunoabsorbed human factor VIII was exposed to 0.025 U/ml of human thrombin and aliquots removed from the mixture at the times indicated.
tive only with factor VIII related antigen. The utilization of mouse monoclonal antibody as an immunoadsorbant to examine factor VIII activation by thrombin has several advantages. Because of the singularly specificity of these antibodies, there is no cross-reactivity with factor VIII:C component of the factor bimolecular complex. These antibodies are selected to recognize distinct antigenic determinants on the factor VIII related protein. With antifactor VIII antisera prepared by standard immunization methods, low levels of antifactor VIII:C reactivity can be a problem and require extensive adsorption to be eliminated. In addition, reactivity from one preparation to another may not be similar. With monoclonal antibody preparations once a clone is identified, large scale production can be readily achieved with standard tissue culture techniques or by preparation of ascites fluid.2

Under the experimental conditions employed, the mechanism for the factor VIII:C generated can best be accounted for by thrombin exerting a proteolytic effect on the immobilized factor VIII:C with the release of a VIII:C fragment. This conclusion is supported by the following: (1) the complete absence of factor VIII:R antigen in the supernatant exclude the VIII:C activity being simply due to the release from the beads of intact factor VIII, i.e., bimolecular factor VIII complex; (2) the lack of procoagulant activity in supernatant when thrombin was added to the beads in the absence of Factor VIII binding (hemophilia A plasma or non-immune beads) eliminates the results owing to a thrombin coagulant effect alone; (3) the lack of procoagulant activity in the supernatant when assayed in a factor IX deficient substrate confirms the specificity of the VIII:C activity generated; and (4) finally, the simultaneous appearance of factor VIII:C and VIII:C antigen can only be accounted for by the proteolytic cleavage of a fragment or fragments containing both of these factor VIII protein functions.

Although the relative amounts of factor VIII:C and C antigen released from beads are discordant from the usual 1:1 ratio in normal plasma, this difference does not necessarily indicate that each function is on separate factor VIII:C protein fragments. This relative increase in activity can be due to the cofactor function of activated factor VIII:C having undergone a probable conformational change resulting in an increased specific activity. This would result in the same number of molecules (as measured by C antigen levels) having a disproportionate procoagulant activity. Conversely, at least two distinct proteolytic fragments may be released, one containing the factor VIII:C and the other only factor VIII:C antigen. If such were the case, the addition of human antifactor VIII antibody to the supernatant samples should have permitted VIII:C activity to be measurable, since antibody would have bound only to the fragment containing the C antigen. Despite a number of attempts, the residual factor VIII procoagulant activity could not be demonstrated by the present authors when this was studied using several human antibody preparations. These data would suggest that factor VIII:C and C antigen as recognized by human antibodies are closely related, if not identical.

References