Distribution of Plasma Fibronectin (Cold-Insoluble Globulin) and Components of the Factor VIII Complex After Heparin-Induced Precipitation of Plasma

By David L. Amrani, Michael W. Mosesson, and Leon W. Hoyer

THE HUMAN PLASMA factor VIII complex has two distinct biological properties. One of them, factor VIII procoagulant (VIII:C) activity, corrects the clotting defect in classical hemophilia A plasma and is associated with an antigen (VIII:CAg) that can be quantified by immunoradiometric assay using alloantibodies. The other, von Willebrand ristocetin cofactor (VIIIIR:RC) activity facilitates ristocetin-mediated platelet aggregation and is presumed to be related to factor VIII mediated adhesion of platelets to damaged vessels. The protein responsible for VIIIIR:RC activity can also be identified immunologically as factor VIII related antigen (VIIIIR:Ag) which precipitates with a heterologous rabbit antiserum to human factor VIII and is reduced in amount in many patients with classical von Willebrand’s disease.

Plasma cryoprecipitate, a concentrate prepared by thawing frozen plasma at 2°C, contains all components of the factor VIII complex and is used for treatment of persons with hemophilia A or von Willebrand’s disease. In addition, cryoprecipitate or a similar plasma fraction also serves as starting material for preparation of factor VIII concentrates of higher specific activity.

Several other plasma proteins and biological activities are also concentrated in this type of fraction. Among these is a plasma protein termed plasma fibronectin (Clg; cold-insoluble globulin). Recent studies suggest that transfusion of cryoprecipitate, the therapeutic value of which is reportedly related to Clg content, may be a useful adjunct in the treatment of septic surgical or trauma patients. Widespread use of cryoprecipitate for the treatment of such conditions could limit its availability for the treatment of hemophilia or for the preparation of other types of factor VIII concentrates.

An economical method for separating the clinically relevant plasma components would provide a solution to this potential problem. We describe here a study in which heparin has been used as the precipitating agent to accomplish these goals. The rationale for such an approach was based on previous data that demonstrated Clg precipitation in the cold by heparin. Our present investigation extends those observations and includes information on heparin-induced fractionation of the plasma factor VIII complex.

MATERIALS AND METHODS

Venous blood was collected from normal individuals, or from a subject (J.G.) with congenital afibrinogenemia, into 0.1 vol of 3.8% Na citrate solution; platelet-poor plasma was prepared from this by centrifugation at 3000 x g for 20 min at 20°C. Outdated pooled ACD plasma (VIII:C ~30–40%) was compared with fresh single donor samples in some experiments. Plasma was also prepared from blood collected directly into Na citrate solution containing Kunitz’ pan...
Creatine trypsin inhibitor (Trasylol, FDA Pharmaceuticals Inc., NY), 2000 units/ml, heparin (Sigma), 2 mg/ml, and Na azide, 2 mg/ml.

Clinical and experimental details concerning the afibrinogenemic subject and his plasma have previously been reported. His plasma fibrinogen level is undetectable by electroimmunoassay (<2 μg/ml; ref. 20) although other plasma coagulation factors (e.g. VIII:C, 128%) and the Clg level (247 μg/ml) are in the normal range. This plasma was stored at -70°C and thawed at either 2°C for preparation of cryoprecipitate or at 37°C for procedures concerning heparin that are described below. Human plasma fibrinogen fraction I-47 was stored frozen at -20°C at a concentration of 21.8 mg/ml in 0.3M NaCl and added to aliquots of the afibrinogenemic plasma to raise its fibrinogen concentration to the normal range (2.5 mg/ml).

Porcine intestinal mucosal heparin (Na salt, grade I, 167 heparin activity units/mg, Sigma Chemical Co., St. Louis, Mo), or heparins having specific activities of 16 and 82.7 heparin activity units/mg (a gift from Cohlfred Laboratories Inc., Chicago, Ill) respectively, were added to citrated plasma at a final concentration of 0.2 mg/ml. In some experiments, additional fractions were removed from the heparin supernatant in inhibitor units/ml and Na azide, 0.2 mg/ml. In some experiments, additional fractions were removed from the heparin supernatant plasma by cryoprecipitation, cold ethanol precipitation, or glycine precipitation. A relatively small amount of precipitate, termed the heparin precipitable fraction (HPF), formed under these conditions and was sedimented by centrifugation at 10,000 × g for 30 min at 4°C. The supernatant plasma was decanted and the HPF washed three times with ice cold 0.05M Tris–Cl buffer, pH 7.2, containing 0.15M NaCl, Kunitz' pancreatic trypsin inhibitor, 100 inhibitor units/ml, and Na azide, 0.2 mg/ml. The washed precipitate was subsequently dissolved at 37°C to the original volume in a 0.25M Tris-phosphate buffer, pH 7.0, containing Kunitz' pancreatic trypsin inhibitor, 100 inhibitor units/ml and Na azide, 0.2 mg/ml. In some experiments, additional fractions were removed from the heparin supernatant plasma by cryoprecipitation, cold ethanol precipitation, or glycine precipitation.

Heparin was adsorbed from solutions with a pellet form of triethylaminoethyl (TEAE) cellulose (Heparsorb, General Diagnostics, Nutley, NJ) by a slight modification of the method of Thompson and Counts. Two pellets were added to each 1.5 ml sample, allowed to swell for 5 min at room temperature, and the mixture was then gently stirred for an additional 10 to 15 min. The tubes were then centrifuged at 2000 × g for 10 min at 20°C and the supernatant liquid removed and placed at 0° to 2°C. Heparin removal was verified by carrying out thrombin clotting time determinations as follows. Test solution, 0.1 ml, was added to 0.1 ml of standard normal human plasma (Citrol, DADE Division, American Hospital Supply, Miami, Fla), the mixture incubated for 2 min at 37°C in an automatic clotting device fibrometer, and the coagulation time measured after addition of 0.1 ml of bovine thrombin (Parke-Davis Co., Detroit, Mich), 5 heparin activity units/ml.

Factor VIII procoagulant activity was determined with a one stage activated partial thromboplastin time assay using pooled fresh frozen normal plasma as a standard. The substrate plasmas were purchased from either DADE Division, American Hospital Supply, Miami, Fla, or George King Bio-Medical Inc., Overland Park, Kan. VIII:CaG was determined by immunoradiometric assay as described. Measurement of VIIIIR:RC activity was made in a Chronolog (Havertown, Pa.) dual channel platelet aggregometer as described by Zucker et al. Briefly, the method involves measuring the steepest slope of the agglutination curve resulting from addition of ristocetin (1 mg/ml, final concentration) (Cutter Biological, Berkeley, Ca) to test solutions containing formalin fixed normal human platelets. The assay was standardized with appropriate dilutions of a pooled normal plasma as a source of VIIIIR:RC. Plasma VIIIIR:RC levels of 3% or less were undetectable in this assay system.

Measurement of VIIIIR:Ag was performed by radioimmunoassay or by electroimmunoassay. Clg was measured by electrophoresis using 125I-labelled rabbit anti-VIIIIR:Ag. Pooled normal human plasma was used for standardizing the VIIIIR:Ag assay. A single donor citrated plasma of known Clg concentration was used to standardize the Clg assay. Separation and analysis of the multimeric forms of VIIIIR:Ag was carried out by Na dodecyl SO4 glyoxyl agarose electrophoresis using 125I-labelled glyoxyl agarose electrophoresis using 125I-labelled rabbit anti-VIIIIR:Ag.

RESULTS

Consistent with previous reports, we found that a final plasma heparin concentration of 0.15–0.25 mg/ml was optimal for forming the HPF regardless of the specific anticoagulant activity of the particular heparin preparation. At the heparin concentration used in this study, 0.2 mg/ml, a mean of 81% ± 5.6% of the total plasma fibroectin was precipitated (Fig. 1).

Ristocetin-induced agglutination of formalin-fixed platelets is partially inhibited by the presence of heparin and VIIIIR:CA activity cannot be measured at all
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The distribution of VIIIIR:RC activity was somewhat different than that of VIIIIR:Ag in that most (72% ± 12%; n = 11) VIIIIR:RC activity was recovered in the HPF. However, considerable VIIIIR:RC activity (52% ± 17%) was also found in the heparin supernatant plasma (HS). The washing procedure applied to the heparin precipitate did not result in significant change of this activity (± 5%; n = 3).

Ten of fourteen plasmas were analyzed for distribution of VIIIIR:Ag multimers by Na dodecyl SO
glyoxyl agarose electrophoresis (Fig. 2) before and after removal of HPF. Consistent with previous results, VIIIIR:Ag in citrated or heparinized plasma was identified as a series of bands reflecting the multimeric forms of this antigen. In all plasmas studied, the largest multimers were found in the HPF whereas the smaller multimers remained in the supernatant plasma. There was, however, no sharp separation of multimeric forms in these two fractions.

The average VIIIIR:RC activity recovered in the HPF and HS was often considerably greater than that of the plasma from which they had been prepared (viz., mean 124%; range 95% to 160%). This observation was explored further by evaluating the net VIIIIR:RC activity in a 1:1 mixture (v/v) of HPF and HS compared with that of HPF and HS measured individually. [Inasmuch as the volume of redissolved HPF was the same as that of HS or the starting plasma, a 1:1 mixture of HPF and HS serves to reconstitute the relative proportions of VIIIIR:Ag multimeric components (as well as other components of plasma) to that found in unfractionated plasma.] The recovery of VIIIIR:RC activity in the HPF from a single donor plasma (VIIIIR:Ag distribution illustrated in panel B, Fig. 2), measured in duplicate at three different dilutions, was 51% ± 8% whereas the recovery of VIIIIR:RC activity in HS was 59% ± 7%. In contrast to the total recovery of 110% observed when HPF and HS were measured separately, a mixture of these two gave a VIIIIR:RC activity of only 78% (based upon recovery of VIIIIR:Ag in HPF and HS, the expected activity was 89%).

In order to explore the mechanism of incorporation of VIIIIR:Ag, VIIIIR:RC activity, and VIIIIR:CAG in the HPF, we determined the distribution of these components (as well as that of VIIIIR:C activity and Clg) in afibrinogenemic plasma before and after the addition of fibrinogen (Table 1). As expected, both normal and afibrinogenemic plasma formed a heparin precipitable fraction, although the amount of Clg precipitated from the afibrinogenemic plasma was substantially less than that from normal unless the plasma had been supplemented with fibrinogen. In contrast to normal plasma, no detectable VIIIIR:RC activity or VIIIIR:Ag

when heparin is present. In order to circumvent this difficulty, we adsorbed the heparin from plasma and its subfractions with TEAE-cellulose. This resin reportedly does not remove any coagulation factors, with the exception of small amounts of factors IX and X. We confirmed this finding in that following adsorption of unfractionated heparin containing plasma, no detectable changes were found in the levels of VIIIIR:C, VIIIIR:CAG, VIIIIR:RC, and VIIIIR:Ag (or Clg). Adsorbed HPF contained no detectable heparin as assessed by colorimetric assay or by prolongation of the thrombin time of plasma. The adsorbed material contained >95% of the original contents of Clg, VIIIIR:Ag, and VIIIIR:CAG (n = 3).

After formation and removal of the HPF from plasma, virtually all VIIIIR:C activity remained in the supernatant fraction, the mean recovery being 77% ± 24%. The same result was obtained with plasma prepared from blood that had been collected directly into heparin containing solutions, and after heparin-induced precipitation of outdated ACD plasma. Although little VIIIIR:C activity was recovered in the HPF (±1%), 20% ± 6.5% of the plasma VIIIIR:CAG was recovered in the precipitate and 54% ± 13% remained supernatant.

Normal citrated plasma diluted 1/7 with 0.25M Tris–phosphate buffer (pH 7.0) prior to assay (VIIIIR:C level 15%) gave the same clotting time in the one stage procoagulant assay as plasma which had been diluted in the same way with saline–Tris HCl, pH 7.0, or with the veronal buffer, pH 7.3, which is routinely used for dilution in this assay. Thus, the Tris–phosphate buffer in which the HPF was dissolved did not account for the low procoagulant activity measured.

We also investigated whether VIIIIR:C activity in the supernatant plasma could be precipitated by application of standard fractionation procedures such as cryoprecipitation, cold ethanol precipitation, or glycine precipitation. Cryoprecipitate prepared from heparin supernatant plasma (two experiments) contained 88 and 93%, respectively, of the original procoagulant activity. In single experiments on the same heparin supernatant plasma, a cold ethanol precipitate contained 82% whereas a glycine precipitate contained 73% of the VIIIIR:C activity.

As assessed by radioimmunoassay, 34% ± 5.2% VIIIIR:Ag was recovered in the HPF and 52% ± 15% in the supernatant plasma (n = 12). Similar values for supernatant VIIIIR:Ag were obtained by electroimmunoassay measurements (59% ± 15%). In those cases in which aggregation of VIIIIR:Ag in precipitated fractions interfered with formation of precipitin "rockets" it was not possible to measure VIIIIR:Ag accurately by electroimmunoassay.

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was precipitated in the HPF from afibrinogenemic plasma unless fibrinogen had been added prior to heparin precipitation. As had been the case for normal plasma, afibrinogenemic plasma showed the same electrophoretic distribution of VIIIR:Ag multimers as normal plasma, with the same tendency for precipita-

tion of the highest order multimers in the fibrinogen containing HPF. We also investigated whether cryo-
precipitate could be prepared from afibrinogenemic plasma and found that, in contrast to the situation with normal plasma, no visible precipitate developed nor was there any measurable change in the plasma level of VIII:C activity, VIIIR:Ag or Clg following thawing in the cold.

**DISCUSSION**

This study has evaluated the distribution of plasma fibronectin and certain components of the plasma factor VIII complex after heparin-induced precipitation. Consistent with previous findings most of the Clg was recovered in the HPF. About one third of the total VIIIR:Ag was recovered in the HPF, and this amount is somewhat lower than that found in plasma fractions resulting from formation of cryoprecipitate. The quantitative immunologic VIIIR:Ag assay does not distinguish the multimeric forms of the antigen that exist in plasma, the higher molecular weight forms of which are believed to possess the VIIIR:RC activity. The results of our Na dodecyl SO₄ glyoxyl agarose electrophoretic analyses support that contention, in that the HPF, which usually contained most of the VIIIR:RC activity, also contained the larger VIIIR:Ag multimers. By contrast, even though supernatant plasma fractions usually contained more VIIIR:Ag than did the HPF, these fractions expressed lower VIIIR:RC activity relative to the VIIIR:Ag level, consistent with their being depleted of the larger more “active” VIIIR:Ag multimers (Fig. 2).

The VIIIR:RC activity precipitated in the HPF was not merely occluded in this fraction, since repeated cold washing of the precipitate did not result in any appreciable loss of activity. Its presence in the HPF is consistent with an earlier report in which VIIIR:RC activity was found in the HPF from certain dermatological patients.

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**Table 1. Heparin-Induced Precipitation of Clg and Components of the Factor VIII Complex From an Afibrinogenemic Plasma**

<table>
<thead>
<tr>
<th>Plasma Level</th>
<th>HPF % Total Recovered</th>
<th>HS % Total Recovered</th>
<th>+ Fibrinogen HPF % Total Recovered</th>
<th>HS % Total Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII:C Activity</td>
<td>128%</td>
<td>&lt;1*</td>
<td>83</td>
<td>&lt;1*</td>
</tr>
<tr>
<td>VIIIR:Ag</td>
<td>100%</td>
<td>&lt;0.8*</td>
<td>84</td>
<td>&lt;1.5*</td>
</tr>
<tr>
<td>VIIIR:RC Activity</td>
<td>170%</td>
<td>&lt;3*</td>
<td>94</td>
<td>35</td>
</tr>
<tr>
<td>VIIIR:Ag</td>
<td>184%</td>
<td>0.3</td>
<td>87</td>
<td>13</td>
</tr>
<tr>
<td>Clg</td>
<td>247 μg/ml</td>
<td>27*</td>
<td>73</td>
<td>62*</td>
</tr>
</tbody>
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*L* limit of sensitivity of the assay  
*Calculated by difference*
As assessed by a quantitative turbidometric platelet assay, the recovery of plasma VIIIIR:RC activity in the fractions resulting from heparin-induced precipitation (i.e., HPF and HS) was frequently higher than expected. However, a mixture of HPF and HS also expressed less VIIIIR:RC activity compared with the expected. We interpret these differences in VIIIIR:RC activity to be due to variations in the ristocetin cofactor activity of the VIIIIR:Ag multimers. The data suggest that the VIIIIR:RC activity of the smaller VIIIIR:Ag multimers (e.g., such as are found in HS) are not detected when the assay is carried out in the presence of larger multimers (e.g., such as those in HPF).

Almost no VIII:C activity was precipitated with the HPF, and this finding is strikingly different from that occurring in the case of cryoprecipitation, in which VIII:C activity tends to be precipitated along with VIIIIR:RC activity. However, a small but significant proportion of VIII:C-Ag (~20%) was recovered in the HPF, despite the fact that there was very low procoagulant activity detectable (≤1%). The VIII:C-Ag in the HPF might represent molecules that have been inactivated by an enzyme such as thrombin. Since the apparent immunoassayable level of VIII:C-Ag is not significantly altered as a function of interaction with thrombin, we tentatively assume that the observed VIII:C-Ag values reflect the content of this protein in the cryoprecipitate.

The distribution of VIIIIR:RC and VIII:C activities after heparin-induced precipitation was the same for fresh and outdated plasmas and was not changed when blood was collected directly into heparin. Gel sieving experiments suggest that the presence of even relatively low levels of heparin (e.g., 2–3 heparin activity units/ml) favors dissociation of the two biological activities of the factor VIII complex. The recovery of most VIIIIR:RC activity in the HPF and virtually all VIII:C activity in the supernatant plasma, may reflect that dissociative effect.

Our analyses of HPF from a fibrinogendeficient plasma before and after the addition of fibrinogen demonstrate a fibrinogen requirement for heparin-induced precipitation of VIIIIR:Ag and VIIIIR:RC activity. This implies that their incorporation in the HPF reflects an interaction with fibrinogen rather than Clg, and that the larger VIIIIR:Ag multimers are not intrinsically insoluble in the presence of heparin.

As an important corollary to the above finding, no cryoprecipitate is formed from a fibrinogendeficient plasma, thus indicating an absolute requirement for fibrinogen. On the other hand, a heparin precipitable fraction does form from a fibrinogendeficient plasma whether or not fibrinogen has been added (Table 1). Thus, there is a qualitative distinction between cryoprecipitation, which requires the presence of fibrinogen, and heparin-induced precipitation which requires the presence of adequate levels of Clg. In contrast to the above, "cryofibrinogen," another type of cold-insoluble plasma precipitate that contains fibrinogen and Clg, is formed only in the presence of fibrin-fibrinogen complexes and Clg.

VIIIIR:RC and VIII:C activities can be dissociated by several physical techniques. These include sucrose density gradient centrifugation, agarose gel chromatography, and fractionation with crosslinked polyelectrolytes. Although heparin precipitation did not completely separate these two activities, the larger VIIIIR:Ag multimers (and most VIIIIR:RC activity) were found in the HPF, whereas virtually all VIII:C activity remained in the supernatant plasma. The use of heparin to achieve this type of subfractionation may have several intrinsic advantages. These include: 1) heparin can be obtained as a sterile, pyrogen-free material, 2) it is reasonably economical, especially considering that precipitation can also be effected with heparins of low anticoagulant activity (this study, 20,30) such heparins are usually discarded during the purification of higher specific activity material for clinical use, 3) the heparin precipitation technique is simple and seems readily adaptable to large or small scale procedures currently in use for the preparation of factor VIII concentrates, 4) if necessary, heparin can easily be removed from solution by employing ion exchange resins such as TEAE cellulose, 5) heparin reportedly stabilizes VIII:C activity, if this observation proves to be correct, then its presence may increase VIII:C recovery for plasma.

Heparin-induced precipitation can be used directly for precipitating the bulk of Clg essentially depleted of VIII:C activity. Fibronectin-rich plasma fractions such as HPF may be useful for treating such conditions as surgical septicemia and trauma. If such is the case, the ability to effectively separate VIII:C activity from Clg would permit separate production of both components from plasma, and this would eliminate the potential strain on the supply of cryoprecipitate that concurrent demand for both might create.

ACKNOWLEDGMENT

We thank Nancy Mingoia for her secretarial assistance and Dorothy Jesinkey, Ellen Feller, and Carl Carta for their technical assistance. We are grateful to Dr. Harold R. Roberts for making plasma from his a fibrinogendeficient patient available to us, and to Dr. Marjorie Zucker for helpful discussions and advice.
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