The Factor VIII Complex: Structure and Function

By Leon W. Hoyer

Normal human plasma contains a complex of two proteins that are important in hemostasis and coagulation. The factor VIII procoagulant protein (antihemophilic factor) and the factor VIII-related protein (von Willebrand factor) are under separate genetic control, have distinct biochemical and immunologic properties, and have unique and essential physiologic functions. While the nature of their interaction and the details of the biochemical structures remain to be determined, the information now available permits a preliminary understanding of the molecular defects in hemophilia and von Willebrand’s disease.

The importance of factor VIII in hemostasis and blood coagulation is obvious from the clinical problems in the factor VIII deficiency diseases, classic hemophilia, and von Willebrand’s disease. During the past decade there has been intense research interest in these diseases, the two most common hereditary bleeding disorders, and in the properties of factor VIII. These studies have led to an evolving understanding of factor VIII structure and function.

The concept that factor VIII has two distinct biologic functions—coagulant activity and a role in primary hemostasis—was first suggested as an explanation for the dual defect in von Willebrand’s disease. The logical inference that factor VIII is a bifunctional molecule was strengthened by reports that proteins purified from human and bovine plasmas had both factor VIII procoagulant activity and the capacity to interact with platelets in a way that might reflect an in vivo role in primary hemostasis. Subsequent studies have suggested an alternative interpretation, and it is now generally accepted that plasma factor VIII is a complex of two components that have distinct functions, biochemical and immunologic properties, and genetic control. The properties of these components are summarized in Table 1 and the relationship is illustrated in Fig. 1.

One component of the factor VIII complex has antihemophilic factor procoagulant activity and is now usually designated VIII:C. It is inactivated by human antibodies and can be measured (as VIII:CAg) when these reagents are used for immunoassays. The other, larger component comprises the majority of the protein mass, interacts with platelets in a way that promotes primary hemostasis, and can be immunoprecipitated by heterologous antisera. It is usually designated factor VIII-related protein (VIIIIR) or von Willebrand factor since it is reduced in quantity or is qualitatively abnormal in von Willebrand’s disease.

Although it has been suggested that the two components are properties of a single macromolecule, several kinds of data demonstrate the essential differences of the two proteins.

1. The factor VIII procoagulant protein and the factor VIII-related protein are controlled by different genes. Isolated VIII:C deficiency is characteristic of hemophilia, a disease transmitted by X-chromosomal inheritance. In contrast, reduced or abnormal VIIIIR is found in von Willebrand’s disease, and the inheritance pattern is that of an autosomal gene.

2. The two proteins can be separated by chromatography or centrifugation in high ionic strength buffers (1 M NaCl or 0.24 M CaCl₂). In most studies, the inclusion of protease inhibitors in the buffers does not affect the separation.
Table 1. The Components of the Factor VIII Complex

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<th>VIII:C</th>
<th>The factor VIII procoagulant protein: the antihemophilic factor.</th>
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<td>The procoagulant property of normal plasma that is measured in standard coagulation assays.</td>
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<td>Factor VIII procoagulant antigen (VIII:C)Ag</td>
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<td>Antigenic determinants closely associated with VIII:C. Measured by immunoassays carried out with human antibodies.</td>
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<th>VIIIR</th>
<th>The factor VIII-related protein: the von Willebrand factor.</th>
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<td>A large polymeric protein that is necessary for normal platelet adhesion and bleeding time in vivo.</td>
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<td>Factor-VIII-related antigen (VIIIR:Ag)</td>
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<td>Antigenic determinants on VIIIR that are detected by heterologous antibodies.</td>
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<td>Ristocetin cofactor (VIIIR:RC)</td>
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<td>The property of normal plasma VIIIR that supports ristocetin-induced agglutination of washed normal platelets.</td>
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(3) The plasma concentration of the two proteins, measured by both function and immunoassays, vary independently under certain conditions, the most striking being the posttransfusion period in patients with von Willebrand's disease.1

(4) The two proteins have different antigenic determinants. Factor VIII procoagulant activity is inactivated by human antibodies from multitransfused hemophiliacs and patients with spontaneous inhibitors; ristocetin cofactor activity (VIIIR:RC) and the bleeding time are characteristically normal in these patients. Each protein can be measured by an immunoassay that is independent of the other protein.14,15 Human anti-VIII:C coupled to Sepharose remove VIII:C (and VIII:C) from plasma, but there is no change in VIIIR:Ag levels.16

(5) The biologic properties of the two proteins are also independent. The procoagulant protein retains full VIII:C activity in the virtual absence of VIIIR (i.e., an VIII:C/VIIIR:Ag ratio of 12,600:1),17 and the factor-VIII-related protein can have VIIIR:RC activity in the absence of detectable VIII:C.18

The two components of the factor VIII complex do interact, however. Their concentrations vary together under most normal, stressful, and pathologic situations,19 and standard purification methods separate the intact (two component) factor VIII complex from other plasma proteins. This interaction will be considered in detail after a description of the properties of the two components.

FACTOR VIII PROCOAGULANT PROTEIN:
ANTIHEMOPHILIC FACTOR

Biochemical Properties

At the present time, there is little published information about the biochemical properties of the factor VIII procoagulant protein, per se. With few exceptions, studies of VIII:C function have been carried out with the intact factor VIII complex, and it is only recently that the factor VIII procoagulant protein has been characterized after separation from VIIIR as well as from the other plasma proteins.13,20,21

Bovine VIII:C has been purified approximately 300,000-fold from plasma by Vehar and Davie.20 Although the G-200 gel filtration properties of the protein with VIII:C activity suggested a molecular weight of 250,000–300,000, analysis in sodium dodecyl sulfate/urea-polyacrylamide gel electrophoresis identified a triplet of protein-staining bands with...
molecular weights of 85,000, 88,000, and 93,000. The electrophoretic properties did not change when purified VIII:C was reduced with 2-mercaptoethanol, but proteolytic cleavage to smaller proteins could be accomplished by incubation with thrombin, purified factor Xa, or activated protein C. The purified bovine VIII:C had no platelet-aggregating activity. Antibodies to the VIII:C protein raised in rabbits inhibited bovine VIII:C procoagulant activity but had no effect on the platelet-aggregating activity of bovine plasma. This observation strongly suggests that the coagulant protein is separate from the protein responsible for platelet-aggregating activity.

Human factor VIII procoagulant protein has not been purified to homogeneity and it may be difficult to obtain the necessary volume of human plasma that has been collected in a way that reduces the likelihood of VIII:C modification in vitro. A major unresolved problem in VIII:C purification is the poor yield obtained with all standard methods. For example, only 0.4 mg of bovine VIII:C protein was obtained from each 125-liter batch of specially collected bovine plasma, an overall VIII:C recovery that was estimated to be 1%.

We have recently completed studies of VIII:C separated from VIIIIR and other human plasma proteins by an immunoadsorbent technique. While this VIII:C is not stable in the absence of added bovine serum albumin or similar proteins that prevent loss of VIII:C from very dilute solutions, the preparation can be studied by both VIII:C functional assays and VIII:C:Ag measurements to determine properties of VIII:C when it is separate from VIIIIR. Although it is difficult to exclude the possibility that the protein was modified during purification, protease inhibitors were present during the procedure, and the purified VIII:C had the same ratio of functional to immunologic activity, as did the plasma from which it was prepared.

The estimated molecular weight of the human factor VIII procoagulant protein separated in this manner is 285,000. This value has been calculated from the properties of VIII:C on Sephadex G-200 gel filtration (Fig. 2A), from which Stokes' radius can be estimated by comparison with standards, and sucrose density gradient centrifugation (8.2S). These properties of unactivated VIII:C are similar to those recently obtained for bovine factor V by Nesheim and coworkers. The correspondence is not surprising since the cofactor role of factor V in prothrombin activation is like that which VIII:C plays in factor X activation. Both proteins are highly asymmetric molecules that are activated by thrombin proteolysis.

Factor VIII procoagulant activity is not inactivated when the intact factor VIII complex is incubated with reducing agents, e.g., 0.05 M 2-mercaptoethanol. This stability is striking when compared to the rapid loss of ristocetin cofactor activity in these experiments. Intact thiol groups do appear to have an important role in VIII:C function, however, and a variety of thiol inhibitors, including the specific reagent, p-chloromer-
curibenzoic acid, inactivate VIII:C.\textsuperscript{23} Factor VIII procoagulant activity is also affected by pH and calcium concentration. VIII:C is most stable between pH 6.9 and 7.2, and a marked loss occurs below pH 6 and above pH 8. The very low VIII:C content of EDTA and ion-exchange resin-treated plasmas indicate that plasma cation concentration is also important.\textsuperscript{24}

Although neither human nor bovine VIII:C has been purified in sufficient quantity for carbohydrate analysis, there is indirect evidence in both cases that the molecule contains carbohydrate residues. Concanavalin-A–agarose binds the purified VIII:C in these preparations, and VIII:C procoagulant activity is eluted by the sugar, \( \alpha-D \) glucopyranoside.\textsuperscript{17,20}

The limited success of VIII:C purification has made it necessary to express the plasma content of this protein by reference to standardized pools of human plasma collected and stored in a way that reduces the likelihood of VIII:C activation or loss. Thus, all VIII:C and VIII:CAg values are arbitrary measures that express a ratio. They do not have any molecular interpretation at the present time. One can, of course, estimate the amount of protein that corresponds to a “normal plasma level” of 1 U/ml. From measurements of the intact human factor VIII complex\textsuperscript{18,25} and the proportion of protein that is VIII:C,\textsuperscript{6} the value is approximately 50 ng/U. A similar value can be obtained from the specific activity of apparently homogeneous bovine VIII:C (4500 U/mg).\textsuperscript{25} It must be recognized, however, that the estimated plasma concentration, 222 ng/ml, is incorrect if the purified VIII:C includes protein that has been either activated or inactivated.

**Immunologic Properties**

Human anti-VIII:C, obtained from multitransfused hemophilic patients that develop inhibitors and from rare individuals who form autoantibodies that inactivate VIII:C, do not form detectable immunoprecipitates with VIII:C or with the factor VIII complex. Nevertheless, these sera can be used to detect VIII:C antigen determinants by antibody neutralization assays\textsuperscript{26} and by more sensitive immunoradiometric assays for VIII:CAg.\textsuperscript{15,27-29}

VIII:CAg immunoassays require radiolabeled human anti-VIII:C that has been purified by preparation of immune complexes followed by their separation and dissolution at low pH. The antibodies have been obtained from high-titer sera (greater than 1000 Bethesda units/ml), and there does not appear to be any consistent difference between hemophilic and spontaneous anti-VIII:C. To date, similar results have been obtained with two different assay methods: fluid phase and two-site solid-phase separations.\textsuperscript{15,27-29} The sensitivity of VIII:CAg assays is 0.01–0.03 U/ml in most laboratories, and the coefficient of variation for these assays is ca. 10%.\textsuperscript{15}

In general, there is excellent correlation between plasma factor VIII procoagulant activity and VIII:CAg content (Fig. 3). VIII:CAg determinants are more stable than VIII:C activity, however, and this is striking in the case of serum in which the VIII:CAg values are 60%–80% of those in the corresponding
plasma, even though there is no residual VIII:C activity. Careful studies carried out with purified VIII:C and human α-thrombin demonstrated a dose-dependent loss of VIII:CAg reactivity at thrombin concentrations below 0.1 NIH U of thrombin/ml—to 60% of the original value—and qualitative changes in VIII:CAg determinants upon exposure to higher thrombin concentrations. These experiments probably overestimated the effect of thrombin on VIII:C, since they were carried out in purified systems and the usual plasma protein inhibitors were not present. It is clear that thrombin has a detectable effect on VIII:CAg, but that this measure is much more stable than procoagulant activity.

**Synthesis**

Despite many investigations, the site of VIII:C synthesis is not known. Both transplantation and perfusion studies strongly suggest that VIII:C is released by the liver under some conditions. These studies, carried out using standard procoagulant assays, are not definitive, however, and it will be important to verify these observations by immunoassay data and protein synthesis studies. Even though there is strong suspicion that the liver plays an important role in VIII:C production, the normal or increased VIII:C values in severe hepatic disease strongly support the concept that there are extrahepatic sources of this protein. In any case, it is not yet known what cell type is responsible for VIII:C synthesis.

**Function**

It is generally agreed that VIII:C accelerates blood coagulation by its cofactor role in the enzymatic activation of factor X by factor IXa. In the presence of phospholipid and calcium, VIII:C markedly enhances this reaction; in the absence of factor IXa, it does not have any intrinsic capacity to activate factor X. Native VIII:C may not participate in this reaction, however, and VIII:C activity is enhanced when plasma or factor VIII concentrates are incubated with dilute thrombin. It is likely, in fact, that thrombin activation is essential for VIII:C activity.

It has been presumed that thrombin activates VIII:C by a proteolytic modification, and this effect has now been demonstrated. Incubation with thrombin appears to cause a cleavage in each of the protein chains of bovine VIII:C, and thrombin-activated human VIII:C has gel filtration and ultracentrifugation properties of a 116,000-dalton protein, in contrast to the 285,000 value calculated for the unactivated molecule (Fig. 2B). Factor VIII procoagulant activity is inactivated by higher concentrations of thrombin—or more prolonged exposure to the enzyme—and a further shift is noted in the gel filtration properties of the (nonfunctional) protein measured by VIII:CAg (Fig. 2C). Thus, thrombin-mediated activation and inactivation are associated with proteolytic modifications of VIII:C structure.

**FACTOR VIII-RELATED PROTEIN: VON WILLEBRAND FACTOR**

**Biochemical Properties**

As the bulk of the factor VIII complex consists of factor VIII-related protein (VIIIIR, von Willebrand factor), data obtained for bifunctional factor VIII reflect VIIIIR properties. In all of these studies, the most striking property of purified factor VIII is its very large size. Agarose gel filtration separations suggested a molecular weight greater than 106 and the molecular weight obtained by sedimentation equilibrium studies carried out in 6 M guanidine was 1.12 x 106. Although highly purified human factor VIII is not dissociated by 6 M guanidine or 1% sodium dodecyl sulfate (SDS), subunits can be detected when VIIIIR is reduced with 2-mercaptoethanol or dithiothreitol. A single band is detected on SDS polyacrylamide gel electrophoresis, and the subunits have an estimated molecular weight of 195,000–240,000.

Studies carried out with highly purified factor VIII may be misleading, however, for they examine only the small fraction of the molecules (ca. 5%–10%) that are not lost during the series of separations. It is now apparent that factor VIII-related protein is, in fact, a heterogeneous population of multimers that have a range of molecular weights from ca. 850,000 to over 12 x 106. This property first became apparent when the technique of crossed immunoelectrophoresis identified VIIIIR heterogeneity. Zone electrophoresis in agarose did not resolve the different forms, however, and the population of multimers was not recognized until VIIIIR was examined in agarose or agarose/acrylamide gels in the presence of sodium dodecyl sulfate. These studies of porcine and human VIIIIR were carried out with purified materials, and it was possible that the apparent multimeric pattern could have been caused by aggregation that occurred during purification. Subsequent analysis of unmodified normal human plasma has demonstrated that VIIIIR circulates as a population of very large multimers and that the size distribution is not an artifact induced by purification methods, freezing, or calcium chelation (Fig. 4).

The smallest polymers detected in normal human plasma have an apparent Mr of 0.85 x 106. This would appear to be a disulfide-bonded tetramer of the basic ca. 200,000 subunit. The larger forms have Mr, indicat-
Fig. 4. The polymer pattern of human plasma VIII-related antigen analyzed by SDS-agarose electrophoresis. The migration of IgM and IgM polymers is indicated on the left and their molecular weights are noted. The VIII:C in plasma samples was identified by autoradiography after incubation with 125I-labeled rabbit anti-VIII:Ag according to the method of Hoyer and Shainoff. From the left, these plasmas are from a normal individual and patients with severe von Willebrand's disease (VIII:Ag < 0.01 U/ml), severe hemophilia, type I von Willebrand's disease, and type II von Willebrand's disease.

ing that they are composed of an integral number of these tetramers, i.e., they have $M_r$ of $1.7 \times 10^6$, $2.5 \times 10^6$, $3.4 \times 10^6$, etc. (Fig. 4). Serum obtained from blood clotted in glass tubes at 37°C has the same population of VIIIIR. As many as 8 separate bands can be detected in most normal plasmas, and there is in addition a population of poorly resolved VIIIIR with $M_r$ of ca. 8-12 $\times 10^6$.

Purified human VIIIIR has also been analyzed by standard biochemical methods. It is a glycoprotein containing 5%-6% carbohydrate, and hexose, hexosamine, and sialic acid have been specifically identified. The amino acid composition has also been determined in these studies: methionine, tyrosine, and tryptophane values are relatively low and there are no free sulfhydryl groups.

The amount of VIIIIR protein in plasma has been calculated from the specific activity of highly purified factor VIII and from immunoradiometric assays of plasma in which purified VIIIIR was used as a standard. The estimated values for VIIIIR protein in normal human plasma have been between 5 and 10 $\mu$g/ml. This is approximately 100 times greater than the concentration of VIII:C protein.

**Immunologic Properties**

Rabbits immunized with purified human factor VIII form useful immunoprecipitating antibodies. Although the sera vary in their ability to inactivate VIII:C, they all form immunoprecipitates with VIIIIR and they inactivate plasma ristocetin cofactor activity as well as other measures of VIIIIR-platelet interaction. They are monospecific in immunoprecipitin assays if prepared with sufficiently purified factor VIII, but absorption with VIIIIR-depleted plasma fractions is often necessary. A number of different assays have been used to detect and quantify this factor VIII-related antigen. Laurell electroimmunoassay, counter immunoelectrophoresis, and radioimmunoassay all give similar results, and antisera prepared in different laboratories have had generally consistent properties in these immunochemical assays.

These rabbit antisera vary in their effect on human VIII:C activity. The properties of the material used for immunization appear to be very important in this regard, and the VIII:C content depends on the purification method. Although small amounts of anti-VIII:C may be present in some sera (in addition to the anti-VIIIIR:Ag) they do not affect the immunochemical measurements. The interaction of anti-VIIIIR:Ag with the intact factor VIII complex will also inactivate VIII:C, and anti-VIIIIR:Ag coupled to agarose removes both VIIIIR and VIII:C from plasma.

In general, there is a good correlation between the factor VIII procoagulant activity and the factor VIII-related antigen concentration in normal human plasmas (Fig. 5). Parallel increases in VIII:C and VIIIIR:Ag have been noted in plasmas from patients with a wide range of nonhematologic diseases and from normal individuals subjected to physiologic stimuli.

**von Willebrand Factor Activity**

Factor-VIII-related protein has a central role in normal platelet function, a property that has been designated "von Willebrand factor" activity because it is deficient in patients with von Willebrand's disease. The prolonged bleeding time in these patients is presumed to be due to the reduced plasma VIIIIR content and it is corrected by transfusion of VIIIIR-rich cryoprecipitate. Two in vitro platelet assays are usually abnormal in von Willebrand's disease: ristocetin-induced platelet agglutination and retention of
platelets in glass bead columns. Both are corrected when purified normal factor VIII is added to blood of a patient with severe von Willebrand's disease. Although the way in which a prolonged bleeding time is a consequence of the reduced level of factor VIII protein is not yet known, there is a good correlation between this abnormality and reduced levels of plasma VIIIIR:Ag and ristocetin cofactor measurements.

The use of ristocetin for in vitro assessment of VIIIIR function has become widely adopted in a rather short period of time. The initial observation—reduced or absent platelet aggregation when ristocetin was added to platelet-rich plasma (PRP) from patients with von Willebrand's disease—provided a simple measure that many laboratories have incorporated into the routine evaluation of patients with bleeding disorders. Unfortunately, the assessment of ristocetin-induced aggregation in patient PRP has limitations as a diagnostic technique. In addition to its qualitative nature, normal or reduced aggregation at one or more ristocetin concentrations, it may be falsely positive in some patients with primary platelet disorders and it is not sufficiently sensitive to detect mild or moderate von Willebrand's disease. While abnormal aggregation in von Willebrand's disease is the consequence of a plasma deficiency and can be corrected by addition of normal VIIIIR, the phenomenon also requires the presence of a normal platelet surface protein that is deficient in Bernard-Soulier syndrome.

Ristocetin-induced platelet aggregation is more critically examined by assays in which dilutions of plasma are tested with washed normal platelets and a fixed concentration of ristocetin. These ristocetin cofactor assays can be done with freshly washed platelets or with formaldehyde-fixed platelets that remain satisfactory as test reagents for several weeks if kept in the refrigerator. The rate of ristocetin-induced aggregation is related to the amount of plasma that is added, and the value can be obtained from the aggregometer tracing or by measurement of the time required by detectable platelet agglutination. The different methods give similar results under most conditions.

Although there is a good correlation of ristocetin cofactor activity in vitro and presumed von Willebrand factor activity in vivo, as judged by freedom from abnormal bleeding and by bleeding time measurements, there are exceptions. For example, the VIIIIR:RC value may become normal in von Willebrand's disease during pregnancy and in inflammatory states, or after transfusion with factor VIII, even though the bleeding time remains prolonged. In addition, patients with a variant form of von Willebrand's disease have long bleeding times in spite of low-normal ristocetin cofactor values and increased reactivity when ristocetin is added to their platelet-rich plasma. Thus, the value of ristocetin cofactor assays as in vitro measures of VIIIIR function must not
obscure the fact that they do not always reflect in vivo biologic function. In this regard, it should also be emphasized that VIIIIR:Ag and ristocetin cofactor assays measure different properties of the VIIIIR protein. While immunoassays detect all VIIIIR molecules with specific antigenic determinants, the protein does not always have biologic activity. Moreover, artifactualy increased immunoassay values are obtained for the smaller VIIIIR polymers when they are compared to whole plasma standards by the Laurell electroimmunoassay method. In contrast, ristocetin cofactor measurements only identify VIIIIR protein that can interact with platelets, and this capacity is limited to the larger polymers. Thus, plasmas or purified proteins that lack the larger VIIIIR polymers will have a very low ratio of VIIIIR:RC to VIIIIR:Ag, and, conversely, material that is relatively enriched in large forms will have higher values for ristocetin cofactor activity than VIIIIR:Ag, when compared to the whole plasma standard.

Radiolabeled purified VIIIIR binds specifically to platelet membranes, and it has been suggested that discrete receptor sites are present. Ristocetin, which also binds to the platelet membrane, apparently makes this platelet receptor available to VIIIIR and enhances its binding. In fact, it has been shown that increasing concentrations of ristocetin cause more VIIIIR molecules to bind to the platelets. A correlation between VIIIIR binding to platelets and ristocetin-induced platelet aggregation has also been demonstrated. VIIIIR polymer heterogeneity complicates these analyses, however, for it is now recognized that only large VIIIIR bind to platelets in the presence of ristocetin.

Several kinds of data suggest that VIIIIR carbohydrate is important in the factor-VIII-platelet interaction and in ristocetin-induced aggregation. Initial studies indicated that sialic acid removal reduced ristocetin-induced platelet aggregation by 65%, other studies found no change in reactivity even though the sialic acid was removed. The latter studies suggested that oxidation of the penultimate galactose modified ristocetin-induced platelet aggregation, and there was a 90% reduction of VIIIIR:RC function when these residues were oxidized. Subsequent reversal of the reaction by galactose reduction caused full regeneration of VIIIIR:RC function. No change in VIII:C activity was noted when intact factor VIII complexes were modified in a way that removed or oxidized these carbohydrate groups.

Carbohydrate residues are also important in VIIIIR intravascular survival. The asialo-derivative is cleared by the rabbit liver with a T1/2 of 5 min; the value for normal VIIIIR is 240 min. Other studies have demonstrated that the rabbit liver lectin that specifically binds asialoglycoproteins binds to the asialo-derivative, not the native or asialo-agalacto-VIIIIR.

Synthesis

Immunofluorescent studies have identified VIIIIR:Ag in endothelial cells of arteries, arterioles, capillaries, and veins throughout the body, as well as in megakaryocytes and platelets. In addition, both VIIIIR:Ag and VIIIIR:RC have been identified in the medium from cultured human umbilical cord endothelial cells. Direct evidence of VIIIIR synthesis by endothelial cells has also been obtained in tissue culture. Factor VIII coagulant activity was not detected in these culture media, however. While it is possible that VIII:C might have been inactivated by a protease present in the media, VIII:C:Ag was also undetectable. Thus, either the culture conditions and/or cell source (umbilical cord veins) are unsatisfactory for VIII:C synthesis or VIII:C is synthesized by a different cell type.

THE INTERACTION OF VIII:C and VIIIIR IN THE FACTOR VIII COMPLEX

Although VIII:C and VIIIIR have very distinct properties, it would be an oversimplification to suggest that they have no relationship and that they are simply two proteins that happen to copurify. Several observations indicate that in plasma they interact through noncovalent bonds to form a complex. For example, the concentrations of the two proteins are closely correlated in normal plasma and in most (nonhematologic) disease states (Fig. 5). In contrast, there is no correlation of either protein with factor V activity. The interaction between the components remains intact when VIIIIR interacts with heterologous antibodies (e.g., rabbit anti-(whole) factor VIII) and VIII:C is included in the immune complex. Immuno-precipitates obtained with rabbit anti-VIIIIR have coagulant activity and elicit anti-VIII:C when injected into other rabbits. Moreover, anti-VIIIIR coupled to agarose removes both VIII:C and VIIIIR from plasma. If VIII:C and VIIIIR did not interact, one would not expect the VIII:C to remain with the immunoadsorbent. An alternative reason for a loss of VIII:C from the plasma, direct binding to unrecognized anti-VIII:C in the rabbit antiserum, can be discarded since the VIII:C can be recovered from the beads by a modest increase in the ionic strength that does not affect immunologic reactions.

Human antibodies to factor VIII have a different effect. Although agarose-bound antibodies remove VIII:C procoagulant activity (and VIII:C:Ag) from plasma, plasma VIIIIR:Ag and ristocetin cofactor activity are unaffected. The difference between the human and the heterologous antibodies in these exper-
THE FACTOR VIII COMPLEX

In hemophilia, the amount of VIIIR removed with VIII:C is very low (2%-10% of normal). Thereafter, quantitative immunoassays were carried out with rabbit antisera to human factor VIII. These studies identified (by quantitative immunoprecipitin measurements) normal levels of "factor-VIII-like protein" in all hemophilic plasmas. Moreover, all hemophilic plasmas neutralized the VIII:C inactivating properties of this rabbit antiserum. Plasmas from patients with severe von Willebrand's disease did not form immunoprecipitates nor did they neutralize the anti-VIII:C activity. These studies, and others carried out with heterologous antisera, led to the concept that nonfunctional but immunologically cross-reactive material is present in all hemophilic plasmas. It is now recognized that immunochemical assays using heterologous antisera—whether done by immunoprecipitation, hemagglutination, or radioimmunoassay—detect VIIIR:Ag, not antigens related to VIII:C function. Thus, they do not demonstrate an intact factor VIII complex in hemophilia A, only normal VIIIR:Ag synthesis as one might expect from the normal bleeding time and the normal values of in vitro assays of von Willebrand factor activity. It was subsequently shown that VIIIR purified from hemophilic plasmas cannot be distinguished from normal VIIIR by standard biochemical methods. Thus, VIIIR:Ag measurements can be used as an assay that distinguishes hemophilia from most forms of von Willebrand's disease, but they do not provide any information about the nature of hemophilia. They measure the product of a different (autosomal) gene.

Immunologic study of hemophilia has become possible, however, as human anti-VIII:C have been used in quantitative immunoradiometric assays for VIII:C antigenic determinants. This technique has permitted both qualitative and quantitative evaluation of VIII:C:Ag and a number of studies have been published during the past 2 yr. They have identified several different patterns of VIII:C and VIIIR:Ag in hemophilic plasmas (Fig. 3).

There is no detectable VIII:C:Ag in most plasmas from patients with severe hemophilia (VIII:C <1% of normal). In one-fourth of these plasmas (33 of 134) there are low VIII:C:Ag levels—usually 1%-10% of normal, but rarely as much as 28%—even though there is no detectable VIII:C coagulant activity. 

Variable VIII:C:Ag levels are present in plasmas of patients with mild or moderate hemophilia; usually there is slightly more immunoreactive material than VIII:C. A more extreme difference is observed for the small group of hemophilic plasmas that have normal VIII:C:Ag even though the coagulant activity is low. These plasmas are from the same patients in which

THE FACTOR VIII DEFICIENCY DISEASES

Hemophilia A

Although the low factor VIII concentration in plasma has prevented biochemical studies in classic hemophilia (hemophilia A), immunologic techniques have begun to define the molecular defect. These studies have attempted to distinguish diminished production of normal factor VIII from synthesis of nonfunctional protein.

Two kinds of immunologic studies must be clearly differentiated as this question is considered. The initial work, done with human antibodies by the technique of antibody neutralization, identified "nonfunctional but antigenically cross-reacting AHF-like protein" in 10% of hemophilic plasmas. The plasmas were designated cross-reacting material positive (CRM +), and they are now known to have normal levels of VIII:C:Ag even though VIII:C is very low (2%-10% of normal). Shortly thereafter, quantitative immunoassays were carried out with rabbit antisera to human factor VIII. These studies identified (by quantitative immunoprecipitin measurements) normal levels of "factor-VIII-like protein" in all hemophilic plasmas. Moreover, all hemophilic plasmas neutralized the VIII:C inactivating properties of this rabbit antiserum. Plasmas from patients with severe von Willebrand's disease did not form immunoprecipitates nor did they neutralize the anti-VIII:C activity. These studies, and others carried out with heterologous antisera, led to the concept that nonfunctional but immunologically cross-reactive material is present in all hemophilic plasmas. It is now recognized that immunochemical assays using heterologous antisera—whether done by immunoprecipitation, hemagglutination, or radioimmunoassay—detect VIIIR:Ag, not antigens related to VIII:C function. Thus, they do not demonstrate an intact factor VIII complex in hemophilia A, only normal VIIIR:Ag synthesis as one might expect from the normal bleeding time and the normal values of in vitro assays of von Willebrand factor activity. It was subsequently shown that VIIIR purified from hemophilic plasmas cannot be distinguished from normal VIIIR by standard biochemical methods. Thus, VIIIR:Ag measurements can be used as an assay that distinguishes hemophilia from most forms of von Willebrand's disease, but they do not provide any information about the nature of hemophilia. They measure the product of a different (autosomal) gene.

Immunologic study of hemophilia has become possible, however, as human anti-VIII:C have been used in quantitative immunoradiometric assays for VIII:C antigenic determinants. This technique has permitted both qualitative and quantitative evaluation of VIII:C:Ag and a number of studies have been published during the past 2 yr. They have identified several different patterns of VIII:C and VIIIR:Ag in hemophilic plasmas (Fig. 3).

There is no detectable VIII:C:Ag in most plasmas from patients with severe hemophilia (VIII:C <1% of normal). In one-fourth of these plasmas (33 of 134) there are low VIII:C:Ag levels—usually 1%-10% of normal, but rarely as much as 28%—even though there is no detectable VIII:C coagulant activity. 

Variable VIII:C:Ag levels are present in plasmas of patients with mild or moderate hemophilia; usually there is slightly more immunoreactive material than VIII:C. A more extreme difference is observed for the small group of hemophilic plasmas that have normal VIII:C:Ag even though the coagulant activity is low. These plasmas are from the same patients in which
CRM+ hemophilia can be identified by antibody neutralization assays. Thus, patients with hemophilia have VIII:C deficiency transmitted by X-chromosome inheritance and they have normal VIIIR synthesis and function. Nonfunctional VIII:C-like molecules are synthesized by some hemophilic patients, and plasma concentrations of immunoreactive protein are normal in rare instances. These patients have an X-chromosome mutation that modifies VIII:C structure. The absence of detectable VIII:C protein in the remaining patients may reflect a structural defect that is so severe that antigenic reactivity is lost (as well as coagulant function) or it may indicate that there is no protein in the plasma.

The evolving understanding of the molecular defect in hemophilia has been based on new methods (VIII:CAg and VIIIR:Ag measurements) that have supplemented standard coagulation assays. This has also improved our ability to provide informed genetic counseling for families affected by this disease. It is now widely recognized that hemophilia carrier detection has been facilitated by the combined measurements of VIII:C and VIIIR:Ag, and most (>85%) hemophilia carriers can be identified when the two assays are done in laboratories that have excellent assay quality control and sufficient experience with reference populations of normal and genetically obligate carriers. Carrier women have normal VIIIR:Ag levels; VIII:C is reduced since only half of their X-chromosomes (on the average) direct normal VIII:C synthesis. The maintenance of an abnormal ratio suggests that the physiologic influences that affect the factor VIII complex act by modifying the production, release, and metabolism of the two proteins in a consistent way.

VIII:CAg stability in the presence of amniotic fluid had led to a further advance in genetic counseling, and prenatal diagnosis of hemophilia is feasible by immunofluorescence analysis of fetal blood samples obtained by fetoscopy at 18–20 wk of gestation. In collaborative studies carried out with Dr. M. J. Mahoney of the Department of Human Genetics of Yale University School of Medicine, immunoradiometric assay for VIII:CAg (and VIIIR:Ag as a control protein) has excluded—or accurately identified—hemophilia in utero for 35 fetuses tested through October 1, 1980.

**von Willebrand’s Disease**

Immunologic and biochemical assays have also clarified our understanding of von Willebrand’s disease. In this case, the hemostatic disorder is transmitted by an autosomal locus that affects VIIIR structure and concentration. Qualitative and quantitative VIIIR defects have been identified in this disease; the reduced VIII:C levels appear to be secondary.

In its most frequent form, von Willebrand’s disease is a mild or moderate bleeding disorder in which all of the components of the factor VIII complex are reduced in quantity and the bleeding time is prolonged. Plasma VIII:C and VIII:CAg may be slightly higher than VIIIR:Ag and VIIIR:RC, but the values are usually similar (Figs. 3 and 5). The VIIIR multimer pattern appears to be normal since all of the VIIIR polymers are reduced in quantity. The bleeding time prolongation is variable, but it is usually associated with reduced ristocetin cofactor activity. While these patients all have genetic defects that affect VIIIR, the hemostatic defect may be quite inconsistent and the results of plasma assays and bleeding time measurements may vary considerably from time to time.

Severe von Willebrand’s disease is a distinct (and unusual) condition in which individuals have very low levels of both factor VIII components, a markedly prolonged bleeding time, and a major bleeding diathesis. Family studies often demonstrate that these patients are homozygous offspring of parents with mild, or asymptomatic, von Willebrand’s disease. Although VIIIR:Ag and VIIIR:RC levels are usually less than 1% of normal in severe von Willebrand’s disease (Fig. 5), some VIIIR:Ag can usually be detected by sensitive assay methods.

It is not certain why VIII:C is low in von Willebrand’s disease, for these patients have the genetic capacity to synthesize this protein. Recent studies suggest that normal VIIIR protects VIII:C from inactivation, and it is possible that VIIIR deficiency permits accelerated VIII:C inactivation in vivo. It is also possible that plasma VIIIR levels may, in some poorly understood way, have an effect on VIII:C synthesis or its release into the plasma. It is likely that an understanding of the low baseline VIII:C level will clarify the mechanism of the delayed rise and prolonged survival of VIII:C (and VIII:CAg) after transfusion in von Willebrand’s disease. At the present time, one can simply suggest that normal (transfused) VIIIR may stabilize VIII:C or it may directly influence VIII:C synthesis or release.

Most patients with von Willebrand’s disease have similar levels of the different factor VIII properties—this has been designated the “classical” pattern. Other patients have been found to have nonfunctional VIIIR, and these individuals have normal or slightly reduced VIII:C and VIIIR:Ag, very low VIIIR:RC, and a prolonged bleeding time. The VIIIR:Ag pattern is abnormal on crossed immunoelectrophoresis, and this
has suggested that there is an abnormal and nonfunctional VIIIIR.\textsuperscript{90,91} It is now apparent that the more rapid VIIIIR migration reflects an increased proportion of small VIIIIR and an absence of the largest multimers (Fig. 4).\textsuperscript{45,92} The shift in multimer distribution changes the VIIIIR electrophoretic mobility, since agarose electrophoresis separates large molecules according to size as well as charge. The important consequence of the abnormal polymer distribution is impaired hemostasis in the variant form of von Willebrand's disease. Platelet binding, ristocetin cofactor activity, and bleeding time correction all require the "type I" pattern in which there is a normal polymer pattern and a similar reduction in all components of the factor VIII complex.

A distinction has recently been made between two subtypes of type II von Willebrand's disease and the abnormal VIIIIR polymer patterns are slightly different. In addition, PRP from patients with the "type IIB" disorder has increased reactivity when tested for ristocetin-induced platelet aggregation and plasma VIIIIR:RC is normal or minimally reduced.\textsuperscript{45,58} In contrast, patients with the more common "type IIA" disease have markedly reduced ristocetin cofactor activity and there is no aggregation when ristocetin is added to their PRP.

The importance of carbohydrate groups in VIIIIR function has been considered in a previous section. It is, therefore, not surprising that reduced VIIIIR carbohydrate has been identified in some patients with von Willebrand's disease. VIIIIR subunits prepared from these plasmas do not have detectable carbohydrate when tested after SDS-polyacrylamide gel electrophoresis, and the VIIIIR sialic acid content is reduced.\textsuperscript{93,94} While a carbohydrate abnormality may be responsible for some instances of nonfunctional VIIIIR, another series suggests that it is an uncommon form of von Willebrand's disease.\textsuperscript{95}

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