METHODS IN HEMATOLOGY

# THE HEMOPHILIAS

Edited by

## ARTHUR L. BLOOM

CHURCHILL LIVINGSTONE

# The Hemophilias

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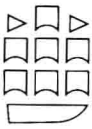

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# The Hemophilias

## METHODS IN HEMATOLOGY

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# Preface

The object of this series is to provide authoritative accounts of methods for the study of blood and its disorders that will be of value in both a hospital and research context. In attempting to apply this principle to hemophilic disorders, I have tried to guide a course between consideration of standard screening tests, which are already excellently described in existing practical texts, and more specialized methods which have little general application in clinical research or practice. As far as the hemophilias are concerned, coagulation assays form the reference basis for many of the other methods. Simple in concept, their practice is fraught with pitfalls for the unwary and, although they are widely used even in modest institutions, it seemed appropriate to commence the practical accounts in this volume with clear descriptions of these basic methods. The rest of the chapters represent my own personal appreciation of important areas of coagulation and other techniques as they are needed for an understanding of the hemophilic disorders. The spread of the volume is not meant to be comprehensive; hemophilia C (factor XI deficiency) and the contact factors, for instance, are not considered. Rightly or wrongly, I have decided to restrict the volume to the disorders which are more commonly observed on a world-wide basis or which present relatively frequently with severe clinical problems. Many recent advances have been due to the introduction of immunological methods into blood coagulation practice and this development is reflected by the fact that several chapters are devoted to these aspects. Finally, the importance of genetic diagnosis has been emphasized by the inclusion of chapters on carrier detection and antenatal diagnosis. All the authors are recognized authorities and I thank them for entering into the spirit of this unique series with great enthusiasm. Finally, I would like to thank the publishers, Churchill Livingstone, for their pleasant co-operation and for the extraordinary efficiency with which the manuscripts were handled. I hope that readers will consider that the volume amply repays their investment and the hard work of all those concerned.

Cardiff, 1982 A.L.B.

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# 1

# Introduction

*A. L. Bloom*

The inherited coagulation disorders are uncommon conditions with an overall incidence probably of no more than about 10 to 20 per 100 000 of the population. Classical hemophilia (A) is the least uncommon — about 5 to 10 per 100 000, and the incidence of hemophilia B (Christmas disease) is usually quoted as about one-fifth of that of hemophilia A. The incidence of von Willebrand's disease is much more difficult to determine, partly because the underlying molecular defects and hence clinical presentation and laboratory findings are variable, and partly because most patients are heterozygous and tend to be mildly affected compared to hemophilia A and B. The overall incidence of von Willebrand's disease in South Wales appears to be about one-half, that of hemophilia A. Inherited deficiencies of other coagulation factors severe enough to cause a significant tendency to bleed are more uncommon although factor XI deficiency is quite frequently seen in Ashkenazi Jews.[1] Nevertheless, in most communities these latter disorders do not pose a very great clinical or therapeutic problem. This volume will therefore concentrate on descriptions of methods for the study of the three most common members of the hemophilia group — deficiencies of factor VIII (hemophilia A and von Willebrand's disease) and factor IX (Christmas disease).

## NORMAL HEMOSTASIS

It is beyond the scope of this volume to review the hemostatic mechanisms and the coagulation system in detail. For such accounts the reader is referred to standard texts such as that edited by Bloom and Thomas.[2] The systems are outlined here merely as an introduction to the chapters that follow and in order to put into perspective the sophisticated methods that will be described.

### PRINCIPLES OF HEMOSTASIS

The processes involved in the arrest of bleeding from small blood vessels such as capillaries and the smaller arterioles and venules seem to differ to some extent from those that are needed for injury to larger (although perhaps still small) vessels. This is illustrated by the fact that the skin bleeding time is prolonged in thrombocytopenia and platelet-related defects, which are conditions which present with small vessel bleeding — petechiae, intradermal or intramucosal bleeding or purpura, and subcutaneous bleeding such as bruises. On the other hand,

the bleeding time is normal in pure coagulation defects such as hemophilia A and B in which petechiae and purpura do not occur. In these conditions abnormal bleeding occurs from slightly larger vessels causing lumpy subcutaneous bruises, intramuscular hematomata and hemarthroses. This does not mean that the coagulation system does not operate in capillary hemostasis nor that platelets are not involved in the hemostatic processes in larger vessels. The observation merely suggests that the platelets probably are the most important component of primary hemostasis in capillaries whereas the coagulation reactions are paramount in consolidating the platelet and hemostatic plug in secondary hemostasis both in small and in larger vessels.

**Primary hemostasis**

The sequence of events that occurs in primary hemostasis is well known and is initiated with vascular contraction mediated via an axon reflex. Platelets adhere to damaged endothelium or to the subendothelium probably by interaction between the membrane glycoprotein 1 complex[3] and a subendothelial component which is possibly collagen[4] or microfibril elastin[5] mediated through a high molecular weight component of the factor VIII complex.[6] Adherence of platelets or their contact with collagen or other components of the vascular environment of uncertain nature triggers a number of reactions which involve the release of arachidonic acid from membrane phospholipid, the subsequent stimulation of the prostaglandin to thromboxane pathway and the generation of thromboxane $A_2$ which causes platelet aggregation. ADP is released from platelet dense bodies and alterations occur in the intraplatelet content of cyclic AMP which influence platelet aggregation. In the presence of a normal platelet membrane glycoprotein complex IIb, IIIa platelets aggregate with the formation of a hemostatic plug. On normal endothelium, the adherence of platelets and the formation of platelet thrombi is limited by the generation of prostacyclin ($PGI_2$) (see Fig. 1.1).

Except for the role of the factor VIII complex in platelet-vessel wall interaction, a discussion of these reactions is beyond the scope of this volume and for this the reader is referred to other texts.[7] However, many of these other platelet reactions are amenable to study in vitro and it is important to exclude the pre-

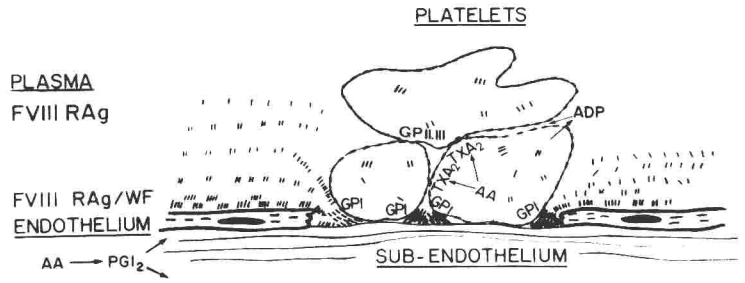


**Fig.1.1** Diagrammatic representation of the role of factor VIII-related antigen/Willebrand factor in platelet vessel wall interaction.
AA = arachidonic acid, $PGI_2$ = prostacyclin, GP = glycoprotein, $TXA_2$ = thromboxane $A_2$

sence of abnormal platelets when considering diagnostic tests for hemophilia A and B and von Willebrand's disease.

**Secondary hemostasis and blood coagulation**

The coagulation sequence is outlined in Figure 1.2 and can be divided into three broad stages; the contact phase, the generation of prothrombin-converting activity and the formation of fibrin. In the initial stages of the system, factor XII is converted into an active form, XIIa, by a surface mediated conformational change and by the action of kallikrein formed from prekallikrein in a cyclical pathway accelerated by high molecular weight kininogen. However, the actual mechanism for the initial activation of factor XII has not been fully elucidated. These reactions are important also in the kinin and fibrinolytic systems. The proteolytic action of factor XIIa is responsible for activation of factor XI, and factor XIa activates factor IX in a two-step reaction.

In the intrinsic coagulation sequence, phospholipid of the platelet membrane plays a vital role in the calcium mediated binding of the γ-carboxyglutamic acid residues of the vitamin K-dependent factors. This capacity of phospholipid brings factor IXa, thrombin-activated factor VIII ($VIII_t$) and factor X into the appropriate spatial configuration so that the latter is activated to factor Xa. In a

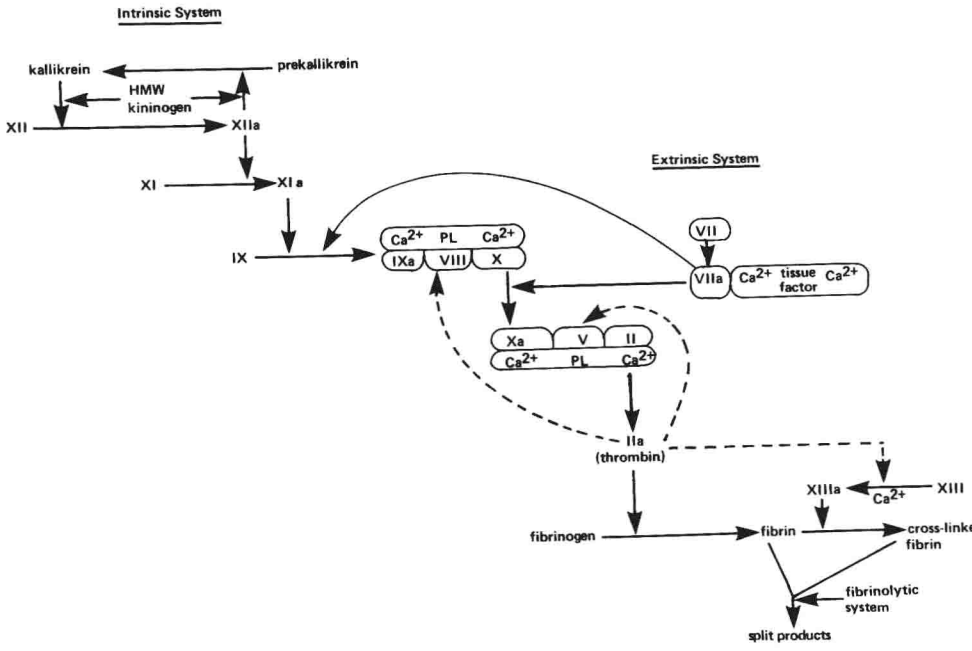


**Fig. 1.2** Simplified scheme of the coagulation sequence. For the sake of this simplicity important control systems such as feed-back mechanisms and humoral inactivators and inhibitors, for example, protein C and antithrombin III, are omitted. The 'extrinsic' and 'intrinsic' systems are separated for discriptive purposes and their many possible points of interaction are omitted. For instance the extrinsic system and factor VII may play a part in the activation of factor IX. The dashed lines indicate the activation activities of thrombin considered to be the most important at the present time.

similar way thrombin–activated platelet–associated factor V ($V_t$), which also contributes a platelet-binding site for factor Xa, promotes the proteolytic activation of prothrombin by factor Xa. These reactions are outlined in Figure 1.2 in which the abbreviation PL indicates both the requirement for phospholipid and the fact that in vivo this is supplied, in part at any rate, by the platelets. Many of the important reactions in blood coagulation thus occur on the platelet membrane where the components not only are brought to the site of injury but may be protected from inactivation by humoral inhibitors such as antithrombin III.

In the final stage of coagulation thrombin attacks arginine-glycine bonds in the Aα and Bβ chains of fibrinogen with release of fibrinopeptides A and B. The resulting fibrin monomer undergoes polymerization and the fibrin polymer is stabilized by cross-linking effected by the transamidase factor XIII.

The extrinsic system is also outlined in Figure 1.2. Tissue factor which is present in cell membranes in most tissues, but not with certainty in platelets, bypasses the earlier stages of the intrinsic system. It is a lipoprotein complex of apoprotein and phospholipid which acts as a cofactor for the activation of factor VII. There are a number of points of interaction between the extrinsic and intrinsic systems and it is important to realize that the coagulation mechanism operates in vivo as a whole and that the intrinsic-extrinsic division is to some extent an artificial descriptive device. Thus factor VII in the presence of tissue factor and $Ca^{2+}$ can also activate factor IX. Factor VII itself is activated by several proteolytic enzymes including factors XIIa, IXa and thrombin but it is not clear which of these reactions is of major importance in vivo. Suffice it to say that the interactions result in the activation of factor X which is one point of convergence with the intrinsic system, and might explain the relative lack of importance of the contact phase of coagulation in maintaining hemostasis in vivo.

In addition to the reactions promoting coagulation, a number of protective mechanisms exist. The central role of platelets in hemostasis helps to localize coagulation reactions to the point of trauma. The formation of fibrin itself helps to contain the effect of thrombin because thrombin is adsorbed to it. Thrombin in turn activates and then inactivates factors V and VIII so that the duration of activity of these factors may correspondingly be reduced. Humoral control mechanisms include antithrombin III and other inhibitors of the serine proteases as well as the possible protective role of the recently described vitamin K-dependent factor protein C.[8,9] In its activated form, protein C inhibits the activity of factor V and VIIIC,[10] but although this reaction may be involved in the combined deficiency of these factors[10,11] there is no evidence that protein C is involved in the pathogenesis of hemophilia A. Finally, when these protective mechanisms have failed, the fibrinolytic system comes into play. It is likely that intravascular deposition of fibrin stimulates local release of plasminogen activator from adjacent endothelium so that the system is poised for protection.

As a result of the above platelet and coagulation reactions and interactions a hemostatic plug is formed and hemostasis is achieved in normal individuals. In due course the hemostatic plug is absorbed or incorporated with the surrounding tissues as healing occurs and the continuity of the endothelium is restored. The formation and fate of the normal hemostatic plug is reviewed by Sixma.[7]

In defects confined mainly to primary hemostasis including thrombocytopenia,

functional platelet defects and heterozygous von Willebrand's disease formation of the platelet plug is delayed but when it does form secondary hemostatic processes are relatively normal so that hemostasis is secured. Thus capillary, mucosal and intra-operative bleeding are characteristic of these disorders and secondary hemorrhage is less common. On the other hand, in hemophilia A and B primary hemostasis is relatively normal and intraoperative bleeding is not usually increased but consolidation of the platelet plug is impaired so that secondary hemorrhage is common. In severe (e.g. homozygous) von Willebrand's disease both types of hemostatic defects may occur.

## FACTOR VIII AND THE PATHOGENESIS OF HEMOPHILIA A AND VON WILLEBRAND'S DISEASE

### The nature of factor VIII

The biochemical nature of factor VIII is uncertain. Originally the term 'factor VIII' was given to that coagulation activity which is lacking in classical hemophilia. The demonstration that 'factor VIII' deficiency occurs in von Willebrand's disease, in which condition there is a deficiency of other hemostatic activities, suggested that even the broad nature of the factor VIII molecule is more complicated than at first envisaged. Briefly, there are three main hypotheses concerning the nature of factor VIII.[12–14]

1. That 'factor VIII' is one type of molecule, although not all may exhibit coagulant activity
2. That 'factor VIII' is two molecules:
   a. factor VIIIC. A 'lower' molecular weight molecule (estimates vary from 85 000–280 000) concerned with coagulation which is reduced or absent in hemophilia A and
   b. a high molecular weight molecule which reacts with heterologous antisera (VIII-related antigen, VIIIRAg) and is concerned with platelet–related activities. It is reduced or abnormal in vWd–factor VIII-related Willebrand factor (VIIIRWF) or ristocetin cofactor (RiCoF).
3. A non-covalently bound complex of these molecules — a hypothesis which seems to find favor at the present time.

The biochemical nature of factor VIIIC such as amino acid sequence is virtually unknown. Vehar and Davie (1980) have recently claimed to have purified it, obtaining 0.4 mg from 120 litres of bovine plasma, and concluded that on gel electrophoresis it forms a triplet with molecular weight of 85 000 to 93 000. According to Hoyer and Trabold[15] immunopurified human VIIIC has a molecular weight of 280 000 when subjected to gel chromatography.

Recent evidence suggests that FVIIIRAg/WF exists as a series of homologous oligomers and that those of highest molecular weight — up to $20 \times 10^6$ show the greatest platelet related activities[16] (see Chapters 5 and 7). The subunit has a molecular weight of about 250 000 and the stable protomer is a dimer or, perhaps, a tetramer, but its amino acid sequence is unknown. Factor VIII-related antigen is synthesized in vascular endothelial cells,[17] but there is no evidence that

VIIIC is synthesized at this site. One possibility is that normally VIIIRAg circulates to another site, e.g. the liver, where it is joined to VIIIC or is altered to acquire the coagulant activity.

**Functions of factor VIII**

*Factor VIIIC* is necessary as a cofactor in the activation of factor X by factor IXa in the presence of calcium and phospholipid. Following generation of a small amount of thrombin the activity of VIIIC increases markedly at first and then declines, so that it is not present in serum. Antigenic determinants of VIIIC (VIIICAg) can however be detected in serum albeit in lower concentration than in plasma.[18]

*VIIIRAg/WF* is necessary for the processes of normal primary hemostasis and interacts with a subendothelial component possibly collagen or microfibril elastin and with platelet glycoprotein 1 in the process of platelet-vessel wall adhesion.

**The pathogenesis of hemophilia A and von Willebrand's disease**

*Hemophilia A* is due to a deficiency of VIIIC activity and may be expected to result from failure to synthesize VIIIC, reduction of synthesis of VIIIC, synthesis of an abnormal variant of the protein which normally exhibits VIIIC activity, or increased production of an inactivator of normal VIIIC activity. Although the last hypothesis was at one time proposed, hemophiliacs respond to transfusion with increased levels of VIIIC consistent with simple deficiency of VIIIC activity. In any case such an inactivator has always been distinguished from the inhibitor antibodies which develop in a small proportion of patients after treatment (see below) and there is no evidence that protein C (or its inactivator) plays any part in the pathogenesis of classical hemophilia.[11] Originally cross-reacting material (CRM) was demonstrated by inhibitor (antibody) neutralization methods in about 10 per cent of hemophilic patients but in a greater number when tested in antibody excess.[19,20] Using an immunoradiometric assay based on an antibody which developed in a hemophilic patient Peake et al[18] demonstrated normal levels of VIIICAg in only one of 30 hemophilic kindred tested. Since hemophilia is inherited as an X-linked recessive characteristic one must assume that the VIIIC molecule or that of a component of its synthetic system is coded on an X-chromosome.

*Von Willebrand's disease* is due to a deficiency or abnormality of VIII-related antigen with secondary deficiency of VIIIC. Studies of the biochemical lesion in von Willebrand's disease have indicated several abnormalities of VIIIRAg in some patients. These include increased electrophoretic mobility,[21,22] reduced carbohydrate content,[23] defective precipitation with concanavalin A[24] — possibly associated with decreased molecular aggregation, abnormal dose-response curves in the immunoradiometric assay for VIIIRAg and a lack of the electrophoretically slow-moving higher molecular weight oligomers[16,25] (see Chapters 5 and 7). These considerations have led to suggestions[14] that von Willebrand's disease may be due to:

1. Reduced synthesis or release of the whole range of oligomers of VIIIRAg. This could be the result of a regulatory gene mutation.[26]

2. Selective reduction of synthesis or release of the higher oligomers of VIIIRAg, e.g. a post-translational defect due to a deficiency of a polymerase or a glycosyl transferase.
3. A true amino acid sequence defect of the subunit of VIIIRAg with resultant failure to polymerize.

In normal individuals there is strong evidence that VIIIRAg/WF (but not necessarily VIIIC) is localized to and synthesized by vascular endothelial cells.[17] Other endothelial cell defects such as reduced production of plasminogen activator have also been reported in von Willebrand's disease[27] as well as morphological vascular abnormalities such as angiodysplasia[28] and telangiectasia.[29] In addition to endothelial cells, VIIIRAg/WF is also present in normal platelets and has been described in the membrane and in the α granules from which it may be released during the release reaction.[14] VIIIRAg may be absent from the platelets in severe (? homozygous) von Willebrand's disease and abnormalities of it at this site have been described in other types of the disease.

It thus appears that von Willebrand's disease may represent a primary synthetic defect of the endothelial cells and possibly of megakaryocytes with reduction or abnormalities of circulating VIIIRAg/WF. This results in reduced levels of VIIIC and impaired interaction of platelet membrane glycoprotein 1 with the subendothelium. From these abnormalities flow the clinical effects of defective primary hemostasis and, in severe cases, the additional problems due to defective coagulation and secondary hemostasis. Possibly because the defect of VIIIRAg/WF seems often to be one of polymerization, which may thus involve all the specific circulating protein, abnormal primary hemostasis may be more apparent than abnormal secondary hemostasis in heterozygotes (i.e. 'dominant' inheritance) since usually there is only modest reduction of procoagulant factor VIII. The variable functional nature of different VIIIRAg/WF polymers may also account for the variable nature of the disease in different kindreds, in different individuals within kindreds and in the same individual at different times, e.g. due to the effects of stress in altering relative levels of the 'normal' component. It may also account for the apparent 'recessive' nature of the disease in some individuals or kindreds. In homozygous von Willebrand's disease VIIIRAg/WF is undetectable by all but the most sensitive methods (see Chapter 5) and levels of VIIIC may be very low. Thus hemarthroses and hemophilic lesions as well as purpura occur in such patients. These considerations are important when interpreting the results of methods to be described in the chapters that follow and are elaborated further in Chapter 7.

## FACTOR IX AND THE PATHOGENESIS OF HEMOPHILIA B

### The nature of factor IX

Factor IX (Christmas factor) is a vitamin K-dependent clotting factor. It is a single chain glycoprotein with minimum molecular weight of about 57 000 and is synthesized in the liver. The amino acid sequence of bovine factor IX has now been determined. There are four main regions of the molecule: the $Ca^{2+}$-binding

region which contains the γ-carboxyglutamic acid residues (Gla-region), the connecting region, the activation peptide region and the catalytic region.[30] The Gla-region shows a high degree of homology with the corresponding regions of other vitamin K-dependent factors.

In the intrinsic coagulation pathway factor IX is activated via two proteolytic cleavages by factor XIa. Initially, an arginine-alanine bond is cleaved to form a two-chain inactive molecule, factor IXα. In the second stage an arginine-valine bond is cleaved resulting in the formation of factor IXaβ consisting of a heavy chain (mol.wt 28 000) linked by disulphide bonds to a light chain (mol.wt 18 000) with release of an activation peptide (mol.wt 11 000). The heavy chain originates from the carboxy terminal end of the molecule and contains the active site; the light chain originates from the amino terminal end and contains the Gla-region. Factor IX can also be activated via the extrinsic system, i.e. the factor VII-thromboplastin complex and in the presence of factor VII, tissue factor and $Ca^{2+}$ it appears that factor IX is cleaved in a manner similar to that due to factor XIa. There is also evidence that bovine factor IX but possibly not human factor IX can be activated by factor Xa but an effect of thrombin or kallikrein is controversial. Factor IX can be activated non-physiologically by Russell's viper venom which splits an arginine-valine bond in the presence of $Ca^{2+}$ (factor IXaα).

**Function of factor IX**

The principal role of factor IXa(α or β) in coagulation is the activation of factor X. In the presence of $Ca^{2+}$, phospholipid and factor VIIIC, factor IXa cleaves an arginine-isoleucine bond in factor X with formation of factor Xa. This is facilitated by the interaction between $Ca^{2+}$ and phospholipid with the vitamin K-dependent γ-carboxyglutamic acid residues in the factor IXa light chain (Gla-region). This reaction is reduced by the presence of acarboxy-factor IX in vitamin K deficiency, or similar molecules may be present as abnormal inherited variants. Methods for their detection will be described in Chapter 9.

In addition to its action in intrinsic coagulation factor IXa may be involved in the activation of factor VII and in the 'cold' activation of the latter factor but its principal function at physiological temperature is in the activation of factor X.

**Pathogenesis of hemophilia B (Christmas disease)**

Hemophilia B is due to a deficiency of factor IX coagulant activity (IXC) and may be expected to result from failure to synthesize factor IX, reduction of synthesis of factor IX or synthesis of an abnormal variant of the protein which normally exhibits factor IX activity. Regions of the factor IX molecule are structurally similar to those of factors II, VII and X, but whereas deficiencies of these molecules are inherited as autosomal traits, hemophilia B is inherited as a sex-linked trait. This does not negate a close similarity between the synthetic mechanisms of these factors, but could indicate, for instance, that abnormalities of hypothetical X-chromosome loci concerned with II, VII and X synthesis or of an autosomal locus concerned with factor IX synthesis are excessively rare. The close relationship between the synthetic mechanisms and/ or functions of these factors is illustrated by the occasional observation of combined deficiency of factor IX with, for inst-