Hemostasis and thrombosis

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INTRODUCTION

Bleeding, caused by vessel wall damage, is halted by the process of hemostasis. Through a series of complicated interactions between the vessel wall, blood cells (in particular blood platelets), and a number of coagulation factors and their inhibitors, a hemostatic plug ensues. The fibrin formed therein is broken down by the fibrinolytic system. Thrombosis can be viewed as the pathological parallel of normal hemostatic plug formation; from a mechanistic point of view, there are a great many similarities.

Bleeding tendencies, as well as thrombotic tendencies, can be caused by inherited or acquired abnormalities of coagulation, platelet function, and fibrinolysis. Although these three processes are intertwined to a considerable extent, in daily practice it is common to make a distinction between platelet-related problems, problems of coagulation, and abnormalities of fibrinolysis. For a long time the laboratory has provided a number of global exploratory function tests (e.g., the bleeding time, the prothrombin time, and the euglobulin clot lysis time). These have some usefulness as screening tests, but they have little sensitivity and, obviously, low specificity. They often do provide some information about the cause of a manifest bleeding tendency. However, they are generally not informative in the case of a thrombotic tendency. Fortunately, the number of possibilities for adequate laboratory analyses has increased considerably over the last decade (Table 14.1). The use of commercial test kits has enabled every reasonably well-equipped laboratory to perform even those determinations that formerly were the domain only of very specialized laboratories.

The foundation for a good laboratory investigation procedure is still laid by means of a thorough medical history and physical examination. Together with the global function tests, these will give indications for necessary additional investigations. Not only from an economic point of view but also to promote a well considered diagnosis, these further investigations should only be performed on the basis of appropriate indications. Apart from diagnostics, laboratory analyses play an important role in the therapeutic monitoring of antithrombotic therapy with heparin and coumarin derivatives.

PHYSIOLOGICAL BACKGROUND

Blood clotting is of necessity a fast, dynamic, and strictly controlled process. It is a massive reaction involving a large number of blood components, both cells and proteins, that should be triggered quickly and efficiently but only when needed. It is as important that clotting does not occur when there is no cause, as it is for it to occur when a vessel is damaged. The guiding principle in the control of this hemostatic process is localization.

Blood clotting is an emergency mechanism. It occurs when a blood vessel is damaged, and blood is exposed to perivascular connective tissue and the contents of damaged cells. Within seconds, processes
Table 14.1. Overview of Commercially Available Hemostasis Tests

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| Chromogenic activity assessment: VII, VIII, IX, X, prekallikrein, endotoxin |
| Antigen determinations (ELISA, Laurell): fibrinogen, II, VII, VIII, IX, X, von Willebrand factor |
| Ristocetin cofactor activity of von Willebrand factor |
| Multimeric pattern of von Willebrand factor |

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<td>TDP (ELISA), specific for fibrinogen and fibrin degradation products</td>
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start that quickly lead to the formation of a seal, or plug, consisting mainly of platelets and fibrin (Sixma and Wester 1977). This plug formation, and therefore the processes involved, remain confined to the damaged area. They are not only limited in place but also in time, as the plug does not grow much beyond the size of the leak. Blood clotting in vitro is thus a phenomenon that is strictly localized temporally and spatially.

Within the scope of this chapter we cannot but touch upon some of the main features, as far as they seem necessary for interpreting the laboratory data.

Components

The proteins, cells, and other substances that make up the hemostatic system form a closely coupled and tightly regulated system. It is therefore difficult to give
a detailed description of the properties of each component separately, without touching on functions of related components. Consequently, only a brief overview of several key components individually will be provided before hemostasis as a whole will be discussed.

**Thrombin.** Thrombin, or factor IIa, is the product of the series of proteolytic activation reactions of the coagulation system. Thrombin has many functions and forms an important link between the various components of the hemostatic process. It is best known for its ability to convert fibrinogen molecules into fibrin monomers that rapidly assemble to form fibrin threads. Thrombin also activates factor XIII, which then has the ability to cross-link fibrin threads to form a rigid network. Thrombin is also a very potent platelet activator, especially when collagen is present as well. The concentration of thrombin necessary for this activation is very low, it may be in the subnanomolar range. Thrombin has several interactions with intact endothelial cells, most of which are antithrombotic. Most important is probably the fact that thrombin is able to form a complex with thrombomodulin, a protein that is present on the endothelial cell membrane. This causes a change of the specificity of the proteolytic action of thrombin so that it loses its procoagulant properties and acquires the ability to activate protein C which, possibly together with the cofactor protein S, forms a potent anticoagulant (Esmon 1989). Reportedly, thrombin also has a number of extrahemostatic actions. It has, among other things, been described as enhancing wound healing by stimulating proliferation of fibroblasts and a number of other cells. Lastly, thrombin plays an important role in the reactions leading to its own formation. By its ability to activate the cofactor proteins factor V and factor VIII, it accelerates thrombin formation by several orders of magnitude (Eaton et al. 1986).

**Platelets.** Blood platelets are small anucleate cells that have an important role in hemostasis. They are activated by a number of substances, among which are thrombin, collagen, and ADP. Activation induces a change in shape from discoid to spherical, accompanied by the formation of pseudopodia. The secretory granules of the platelets then release their contents into the open canalicular system, which communicates with the surrounding plasma. Among the substances released are ADP, for activating neighboring platelets, coagulation factor V, von Willebrand factor, fibrinogen, platelet factor 4, and vasoactive substances like serotonin. Platelets are capable of adhering to proteins of the subendothelial matrix, like collagens, to various plasma proteins like fibrin, von Willebrand factor and fibronectin, and to other platelets. This adherence is mediated by cell surface receptors of the integrin family (Furie and Furie 1988). The platelet surface receptor for fibrin, GPIIb/IIIa, probably has to be activated before functioning, but it is not clear whether this holds true for other members of the integrin family.

Platelet activation by thrombin together with collagen also leads to loss of platelet membrane asymmetry by virtue of the so-called translocate reaction, or flip-flop. This results in the translocation of negatively charged phosphatidyl serine molecules to the outer leaflet of the platelet membrane bilayer. Reactions of the coagulation system are accelerated by several orders of magnitude when occurring on such a membrane surface, as this phospholipid surface lowers the K_m of several reactions of the coagulation system (Zwall and Hemker 1982). Normally, phosphatidyl serine molecules are virtually absent from the outer leaflet of the membranes of cells, which explains why most intact cells do not promote coagulation. Small microvesicles are shed by activated platelets. These microvesicles have lost their membrane asymmetry as well and can thus serve as a surface for the various membrane bound reactions of the coagulation system.

**Vitamin K-dependent Clotting Factors.** The vitamin K dependent clotting factors include factors II, VII, IX, and X. They are serine proteases that undergo a vitamin K dependent posttranslational carboxylation of certain glutamic acid residues to γ-carboxyglutamic acid residues (Nemerson and Furie 1980). They bind in a calcium-dependent manner to negatively charged lipid membranes through these γ-carboxyglutamic acid residues (Mann et al. 1992). Factor VII, when bound to tissue factor, is proteolytically cleaved to factor VIIa by factor Xa and, at a slower rate, by factor IXa. Factor VIIa, when complexed to tissue factor, can activate factors X and IX. Factor VII is present in plasma in a relatively low concentration of 10 nmol/L. It is still a matter of debate whether or not the zymogen factor VII has a physiologically important proteolytic activity or not. If it has, it would be the only clotting factor to have this capability as a zymogen, and it would hint at a plausible theory about the initiation of coagulation. Factor IX, which has a plasma concentration of about 80 nmol/L, can be activated to factor IXa by the tissue factor/VIIa complex and by factor Xa, although this latter reaction probably has no physiological significance. Factor IXa’s main function is the activation of factor X. To form an efficient enzyme, factor IXa complexes with its cofactor, factor VIIIa, on a phospholipid surface. Factor X, present at a concentration of approximately 160 nmol/L, is activated by the IXa/VIIIa complex, or by the VIIIa/tissue factor complex. Factor Xa joins with its cofactor, factor Va, on a phospholipid surface to form the prothrombinase complex, which activates prothrombin. The vitamin K-dependent thrombin precursor, prothrombin, is present in a concentration of around 1.5 μmol/L. All vitamin K-dependent clotting factors retain their γ-carboxyglutamic acid residues when they are activated, except for thrombin. Thus, thrombin does not bind to a phospholipid surface.

**Cofactors.** The three main enzyme complexes leading to the formation of thrombin all consist of a complex of a vitamin K-dependent clotting factor and a protein cofactor on a phospholipid surface (Mann et
Cofactors enhance the catalytic efficiency of their enzymes several orders of magnitude by increasing the k_{cat}.

Tissue factor is a small transmembrane glycoprotein that is found in the plasma membranes of many cells but not normally on the luminal side of the cells of the endothelium. It forms complexes with factor II or VIIa (Nemerson 1988). Unlike the other cofactors, tissue factor does not need to be activated. Monocytes are capable of acquiring tissue factor activity on the outside of their cell membranes when stimulated, e.g., by endotoxin, thus rendering them procoagulant.

Factor VIII and factor V are large proteins with similar structural properties (Kane and Davie 1988). They are cofactors of factor IXa and factor Xa, respectively, and must be proteolytically cleaved by thrombin to their active forms in order to gain cofactor activity. Both bind strongly to a phospholipid surface in a calcium independent manner. Factor VIII circulates in plasma in a complex with von Willebrand factor in a low concentration (1 nM/L). The plasma concentration of factor V is about 30 nM/L, but it is also present in the α-granules of blood platelets.

Physiological Inhibitors of the Coagulation Cascade. Several members of the serine protease inhibitor (or serpin) superfamily (Travis and Salvesen 1983) are able to inhibit the vitamin K dependent serine proteases of the coagulation system. These include antithrombin III, heparin cofactor II, and α1-antitrypsin. Their mechanism of action is that of a pseudosubstrate. Binding is reversible, and it is thought to follow a two-step process, with the formation of an initial loose complex that then proceeds to a tightly bound complex. The rate limiting step is the formation of the initial weak complex, which makes the rate of inhibition slow (k_{cat} equals 1.5x10^{-3} nM/L/min). The dissociation rate of the tight complex is so slow, however, that binding has long been thought to be irreversible. Very slowly, the reaction between serpin and protease terminates in proteolysis, resulting in cleaved inhibitor and active protease. Heparin and heparin-like antithrombotics potentiate the action of antithrombin III by decreasing the dissociation constant of the weak initial complex by several orders of magnitude, without affecting the rate of formation of the tight complex (Hemker and Beguin 1992). They act as catalysts, in that they are liberated from the antithrombin III molecule concomitant with the formation of this latter complex, and are thus available for binding to other free antithrombin III molecules. The endothelial surface contains glycosaminoglycans having a heparin-like action. Patients with abnormal antithrombin III that cannot be potentiated by heparin have been described. The existence of a physiological parallel of heparin action, possibly via these glycosaminoglycans, is hinted at by the thrombotic tendency in such patients.

Tissue factor pathway inhibitor, a Kunitz type serine protease inhibitor circulating in plasma at a concentration of about 2.5 nM/L, is able to form a ternary complex with factor Xa and tissue factor/VIIa, thereby inhibiting both enzymes (Broze et al. 1990). Once sufficient factor Xa has been produced, tissue factor pathway inhibitor is able to completely shut off tissue factor/VIIa activity.

A third inhibitory component is formed by protein C and protein S. Protein C, a vitamin K-dependent, phospholipid binding protein, is activated by the thrombin/thrombomodulin complex on intact endothelium. Activated protein C, together with protein S, is directed against the cofactors Va and VIIa, which are proteolytically inactivated (Esmon 1989).

Fibrin. Fibrinogen consists of two halves, each constructed from three elongated protein chains (the Aα-, Bβ-, and γ-chains) (Doolittle 1984). It circulates in plasma in the shape of an elongated dumbbell with a medial thickening. It is one of the most abundant plasma proteins with a concentration of about 10 μM/L. Thrombin cleaves off the fibrinopeptides A and B (A before and faster than B). The fibrin monomer, thus formed (2(αβγ)) polymerize to double stranded fibrils. Both the formation of the strands and the association to a thicker, branched net occur nonenzymatically by the physicochemical interaction of fibrin monomers. Plasma transglutaminase (factor XIII) catalyzes the formation of transverse covalent linkages between certain lysine and glutamic acid residues, which enhances the stability of the fibrin net considerably. In plasma, factor XIII is complexed to fibrinogen. It is activated by thrombin but only in the presence of fibrin strands, which is to say, when its substrate is already present. Thus, fibrin is not an inert lattice. It also plays an important role in regulating the activity of thrombin, platelets, and fibrinolysis. A significant amount of the thrombin formed during clotting binds to fibrin being generated, where it is protected from inhibitors and hides its enzymatic properties. When fibrin-bound thrombin is released during fibrinolysis, it again exposes its enzymatic activity.

Prostanooids. Prostanooids are a group of substances that mediate many physiological processes. Stimulated platelets synthesize the platelet activator, thromboxane A2, and endothelial cells produce the platelet inhibitor, prostacyclin. Prostanooids originate from polyunsaturated fatty acids through the action of cyclooxygenase. This enzyme is irreversibly inhibited by acetyl salicylic acid. Whether this explains the prolonging effect on the bleeding time and the antiplatelet effect of acetyl salicylic acid is not completely clear. Acetylsalicylic acid has a net antihemostatic and antithrombotic effect, in spite of its inhibition of both thromboxane synthesis in the platelet (antithrombotic) and prostacyclin synthesis in the endothelial cell (prothrombotic). An explanation for this is that endothelial cells (in possession of a nucleus) do and platelets (anucleate) do not have the capability to synthesize new enzymes replacing the irreversibly inhibited ones. The hypothesis that polyunsaturated fatty acids influence hemostasis and thrombosis because they are the raw material from which
prostanoids are derived is attractive, albeit not yet completely proven.

**Reactions**

The process of coagulation is based on the activation of a zymogen by another enzyme, similar to the formation of trypsin from trypsinogen by enterokinase in the duodenum. The now-obsolete cascade theory explained coagulation exclusively through this principle (MacFarlane 1964). The backbone of the coagulation process consists of the activation of prothrombin (factor II) by activated factor X (factor Xa), which itself is generated by activated factor VII. If we represent such an activation by an arrow, this backbone can be depicted as follows:

\[ \text{VIIa} \rightarrow \text{Xa} \rightarrow \text{IIa} \]

Factor VIIa can, in addition to factor X, also activate factor IX, which then is also able to activate factor X,

\[ \text{VIIa} \rightarrow \text{IXa} \rightarrow \text{Xa} \rightarrow \text{IIa} \]

This mechanism can produce a considerable amplification of factor X activation even though the extent of direct activation via factor VIIa is relatively small (Xi et al. 1989a). This scheme differs from the classical coagulation scheme, with its intrinsic-extrinsic dichotomy. The recognition that contact activation of coagulation probably plays no important role in vitro, as well as the discovery of the Josso loop, has led to the demise of this hypothesis.

In purified systems, factor Xa is also capable of activating the cofactors V and VIII (Eaton et al. 1986, Foster et al. 1992). This ability does not seem to play a role in a plasmatic environment, however.

**Enzyme Complexes.** The factors VIIa, IXa, and Xa alone are very slow enzymes. They need accessory components, a phospholipid surface and a cofactor, in order to achieve their full potential (Fig. 14.1) (Mann et al. 1992). The properties of the prothrombinase complex have been investigated in much detail. When factor Xa is bound to a phospholipid surface, the K_M for prothrombin conversion is several orders of magnitude lower than when factor Xa is free in solution (Mann et al. 1992). This means that, without a phospholipid surface, factor Xa would need 40 times the normal plasma concentration of prothrombin to be 50% saturated, whereas in the presence of negatively charged phospholipids it performs optimally, even at the normal plasma concentration of prothrombin. The presence of the cofactor, factor Va, increases the turnover number of the enzyme about a thousandfold (Mann 1987, Nesheim et al. 1979). This means that complete prothrombinase, the complex of factor Xa and factor Va on a phospholipid surface, is a very efficient enzyme; one that converts prothrombin into thrombin so quickly that under normal physiological conditions, the reaction rate is limited only by diffusion of prothrombin to the phospholipid surface containing the prothrombinase complex. In addition to enhancing its turnover number, phospholipid-bound factor Va greatly increases the affinity of factor Xa for the phospholipid surface (Giesen et al. 1991). The other enzyme complexes, factor VIIa with its cofactor tissue factor and factor IXa with cofactor VIIIa, have properties comparable to those of the prothrombinase complex (Mann et al. 1992). Calcium ions are necessary for binding of the vitamin K dependent clotting factors to the phospholipid membrane and for the structural stability of the cofactors Va and VIIa. When blood is collected in citrate or EDTA, the calcium ions are chelated, and thrombin generation is thereby inhibited. Addition of calcium ions again triggers the process.

**Initiation.** Normally, clotting enzymes and cofactors circulate in their inactive form, and no suitable phospholipid bilayer is available on which coagulation reactions can take place. However, small amounts of the activation peptides of the clotting factors can always be measured in human blood, indicating that very low concentrations of activated clotting factors are always present. This basal activity may be important in the initiation of coagulation. When local vessel wall damage occurs, blood is, among other things, exposed to extravascular connective tissue and to tissue factor. Furthermore, cell damage may expose procoagulant phospholipid from the cell interior. It is hypothesized by some groups that the initiation of coagulation results from the complexing of tissue factor and the zymogen form of factor VII which has some proteolytic activity towards factors X and IX. To date this mechanism of initiation has not been proven, and it may well not be important in the light of the steady,
albeit low level, presence of activated clotting factors in circulating blood. Thus, a little factor VIIa is already present, and zymogen form of factor VII, when complexed to tissue factor, can readily be activated by circulating factor Xa.

The other leg of the initiation of the hemostatic process is the adherence of platelets to the revealed subendothelium, with subsequent platelet shape change and release of active substances like ADP and platelet activating factor (Sixma and Wester 1977). These cause binding of other platelets to the first layer of adhered platelets and a primary aggregate starts to form. In addition to platelet cell surface receptors, both fibrinogen and von Willebrand factor are required for adhesion and aggregation. Aggregation, i.e., platelets binding to each other, can be observed in the laboratory by adding thrombin or ADP to platelet rich plasma. ADP-induced aggregation is reversible. If, in such primary aggregates, traces of thrombin are formed, secondary irreversible aggregation ensues. This happens when the usual laboratory conditions apply and most likely also in vitro.

Positive Feedback. The tissue factor/VIIa complex is a rather efficient activator of factor X and IX, but at the time of initiation of coagulation these factors do not yet have available the activated cofactors. Therefore prothrombin conversion is initially a very inefficient and slow process, leading only to trace amounts of thrombin. These concentrations of thrombin are far from high enough to convert massive amounts of fibrinogen into fibrin, but they do lead to small scale activation of the cofactors, factors V and VIII, and of blood platelets. This latter activation leads, among other things, to exposure of a procoagulant phospholipid surface (Zwill and Hemker 1982). Without thrombin activation the adherence and aggregation of platelets is not sufficient for the exposure of negatively charged phospholipid surface. Formation of small amounts of complete prothrombinase and factor Xase enzyme complexes ensues, leading to an increase in the rate of thrombin production, the catalytic efficiency of these complete enzyme complexes being more than five orders of magnitude higher than that of the enzymes alone. From this point, the reaction sequence exhibits a typical positive feedback pattern with a very rapid increase in reaction rates and product formation (Hemker and Kessels 1991). Concentrations of thrombin reach several hundred nanomoles per liter, more than enough for efficient fibrin formation. Thus, with thrombin being the most important physiological activator of blood platelets, and platelets being necessary for thrombin formation of any importance, reactions involving platelets and coagulation are closely interrelated. The classical view of hemostasis as a two-stage process (i.e., first platelet reactions, then coagulation), therefore, represents much too simplified a model.

Inhibitory Reactions. Several inhibitory reaction pathways run through this scheme as well (Esmon 1987). Antithrombin III and several other serine protease inhibitors inhibit thrombin and factor Xa at a slow but concentration dependent pace. The factor VIIa/tissue factor complex falls prey to tissue factor pathway inhibitor after a certain lag time. The activated cofactors may be attacked by activated protein C, possibly accompanied by protein S. Perhaps less obvious, but probably at least of equal importance, are several other inhibitory mechanisms. Dilution of the active factors by blood flow is an important factor that is very difficult to assess since the exact flow conditions at the site of a wound may be very complicated and variable. Competition between reactions should also not be neglected. In vitro, competition may all but completely suppress reactions in vitro, that are clearly demonstrated in purified systems. This is especially true regarding limited resources, such as procoagulant phospholipid surface and enzymes with many substrates, such as thrombin. Rather interestingly in the latter case, serpins, like antithrombin III, have a very low initial affinity for thrombin. Therefore, they do not effectively compete for thrombin in the initial stages of the clotting process when thrombin concentrations are low, so that positive feedback may proceed unhindered.

Control

The myriad of activations, complex formations, and inhibitions involved in the hemostatic process may give the impression of an extremely complicated, not very well-organized, group of reactions that, almost magically, seem to produce the desired effect. Things fall into a little better when one realizes that most of these reactions are localized in space and time.

Space Localization. Hemostasis is needed only at a particular place, the site of vessel injury. This space confinement is reflected in several aspects of the underlying reactions. First, the reactions that form the backbone of the coagulation system all take place with the components, enzyme, cofactor, and substrate bound to a negatively charged phospholipid membrane, a membrane that is not normally accessible. Only when cells are damaged or platelets activated does the negatively charged inner leaflet necessary for the clotting reactions become exposed. All the vitamin K-dependent clotting factors bind to negatively charged phospholipid surfaces. Their activated forms also bind, with the exception of thrombin. Even thrombin is partially confined to the site of injury, though, by loosely binding to the fibrin being generated. Second, platelets contain a number of receptors that cause them to adhere to components of the subendothelial connective tissue. Third, intact endothelium, which surrounds the site of injury, has a number of mechanisms for inhibiting procoagulant reactions. Fourth, blood flow keeps hemostatic reactions localized since it dilutes any activated components of the hemostatic system that drift away from site of injury.

Time Localization. The general idea of time localization is that procoagulant reactions are fast but short-lived and that anticoagulant reactions are slow
but persistent. The character of hemostasis as an emergency process is reflected in the rapid increase of the reaction rates of the procoagulant reactions. This is a consequence of both the fact that coagulation is a multistep process, with the intermediate steps acting as amplifiers, and of the various positive feedback mechanisms.

It is of great importance that the hemostatic reactions at the site of an injury are efficiently shut off after a while but neither too soon nor too late. Thus, inhibitory mechanisms should initially keep a slack reign on the procoagulant reactions but must halt them a little later on. The initial trigger of the coagulation system, the tissue factor/VIIa complex, is blocked within minutes by tissue factor pathway inhibitor, which starts being effective as soon as sufficient factor Xa is present. This, however, does not stop the activation of factor X by the factor Xase complex or the formation of thrombin by the prothrombinase complex. Here, antithrombin III and a number of other serpins, which are all present in a relatively high concentration in plasma, come into play. They first bind their target enzymes in a weak reversible manner. This initial complex is then converted into a nearly irreversible one. Since the rate of inhibition is determined by the speed of the initial complex formation, it is slow, in spite of the high concentration of the inhibitor. Consequently, the inhibitory capacity of the serpins is initially overpowered by the burst of enzymes generated early in the procoagulant reactions. The inhibition rate of the serpins is also linearly dependent on the enzyme concentration, a property that eventually limits the concentration of active enzyme as the inhibitory rate starts balancing the formation rate of the enzyme later in the procoagulant reaction. Another phenomenon that conceivably favors, and later counteracts, procoagulant reactions is impediment of blood flow inside the forming fibrin and platelet clot. Initially, the dilution of active clotting factors is diminished, which has a procoagulant effect. Eventually, though, consumption of the clotting factor zymogens, without their replenishment, leads to a decreasing rate of activation.

**Space and Time Localization.** It is noteworthy that some of the same mechanisms that cause spatial localization also lead to temporal localization. The prime example of this is the occurrence of clotting reactions on negatively charged phospholipid surfaces. As mentioned above, this confines clotting factors to the site of vessel damage. But it also accelerates those reactions quite considerably by effectively increasing the enzyme size to include a relatively large area of phospholipid surface, thereby concomitantly increasing the collision rate. Another example is the absence of blood flow inside a clot. This prevents washout of thrombin and other activated clotting factors, and in this manner produces space localization. As described above, it also has a temporal effect since clotting factor zymogens are being consumed inside this region of stasis.

## BLEEDING TENDENCY

**History and Physical Examination**

In general, a solitary bleeding episode is not a manifestation of a general bleeding tendency but of a local pathology. Only if there are repeated hemorrhages, if large hematomas are seen, or if no local cause can be found, should a bleeding tendency be considered. In the case of platelet or vessel wall defects, mucosal hemorrhages are encountered chiefly (epistaxis, bleeding of gingiva, bleeding of endometrium, hematuria, hemoptysis, and bleeding from the gastrointestinal tract). In the case of coagulation factor deficiencies, large and deep hematomas in muscles or joints are more common. Bleeding is also seen at minor surgery (e.g., dental extraction). Through the medical history, one should try to ascertain the presence and characteristics of the bleeding tendency. Inquiries should be made about earlier dental extractions and tonsillectomy. To be sure, these are “minor” operations, but they demand a lot of hemostasis and are therefore good measures of the severity of the possible underlying disorder. In addition to this, one should enquire about blood loss from other surgical or invasive operations, like punctures, injections, and so on, blood loss after parturition, and blood loss from small wounds. Transfusions can be an indication of the significance of the bleeding. Disturbed wound healing (keloid scar tissue) is seen in cases of factor XIII deficiency. The possibility of artifacts or withheld traumas (self-mutilation, battered-child syndrome) should also be considered. If the hemorrhagic tendency has been present from childhood, a congenital defect should be contemplated, although the inverse does not hold. Unexplained bleeding in a middle-aged person can be caused by mild hemophilia. By means of a focused family history with a family tree, it can be determined whether or not a hereditary defect is involved and how the defect is inherited. A negative family history does not preclude a congenital defect, however, because there is a relatively high frequency (30%) of spontaneous mutations and because clinical symptoms may be lacking in cases of mild hemophilia or in heterozygotes.

A number of drugs can impair hemostasis. The obvious example is acetylsalicylic acid. It is often difficult to elicit the taking of acetylsalicylic acid. It must be realized that the number of preparations containing acetylsalicylic acid is enormous and that its effect is irreversible and detectable until 10 days after its ingestion. Other nonsteroidal antiinflammatory drugs, like indomethacin and ibuprofen, cause a reversible disturbance of platelet function. Apart from medication, inquiries must also be made about intoxications (alcohol, mushrooms, narcotics, chemicals in relation to profession or hobby), whether blood products were administered in the past, and whether there is a possibility of inadequate nutrition. In addition it is essential to know whether a patient receives oral anticoagulants or another kind of antithrombotic therapy.
Bleeding may be the first symptom of diseases of the liver and kidney, (hemato-)oncological diseases, autoimmune diseases, disturbances in gastrointestinal absorption, and various infections. Therefore, it is always indicated to perform a complete physical examination. Specific attention should be given to petechiae, ecchymoses, and hematomas on skin and mucous membranes. The aspect (color, elevated or not), number, size and location should be noted. In the case of a clearly palpable purpura, vasculitis should be considered. Petechiae and multiple small hematomas are especially common with thrombocytopenia, thrombocytopathy, and vasculopathy. Apart from spider nevi, one should also look for other stigmata of liver disease. Extensive hematomas and acute hemorrhage in the absence of significant trauma generally point to coagulation factor deficiencies. Ankylosis, muscular atrophy, and other functional disturbances of the motor system can be caused by old untreated muscle or joint hemorrhages and also suggest coagulation factor deficiencies. Other areas that deserve attention are the aspect of scars (factor XII deficiency) and any signs of hereditary connective tissue diseases that themselves cause an elevated bleeding tendency, like Marfan's syndrome or Ehlers-Danlos syndrome. One should also note the presence of telangiectasia, including in the conjunctivae, on and under the tongue, on the lips, and on the palms of the hands and the soles of the feet, as is seen in Rendu-Osler disease. In general, a bleeding tendency does exist with such vessel anomalies, but an underlying hemostasis defect does not. With extensive hemangiomas, such as are found in Kasabach-Merritt syndrome, thrombocytopenia and other symptoms of intravascular coagulation may be observed.

**Exploratory Laboratory Investigations**

Indications for requesting exploratory hemostasis tests include analysis of a bleeding tendency, intention of a blind biopsy (liver, spleen, kidney) or an endoscopic retrograde cholangiopancreatography (ERCP), suspicion of diffuse intravascular coagulation, vitamin K deficiency, insufficiency of the kidneys or the liver, paraproteinemia, and amyloidosis. Preoperative hemostasis investigation for screening purposes in the case of a negative history does not make sense (Rapaport 1983; Suchman and Griner 1986). The initial investigation consists of the following determinations: hematocrit and other relevant parameters from routine hematological studies, platelet count, activated partial thromboplastin time, prothrombin time, thrombin time, and bleeding time.

**Platelet Count.** In general, platelets are counted in EDTA anticoagulated whole blood, using an automated cell counter. A low platelet count reduces the validity of the procedure, as do a highly elevated number of leukocytes, turbid samples, the presence of many fragmentocytes, the presence of fragments of nuclei in erythrocytes, much enlarged platelets, or the presence of microaggregates of platelets, e.g., caused by inadequate mixing during thrombosis. Pseudothrombocytopenia arises when EDTA causes agglutination of thrombocytes (Lombarts and Delievet 1988). This is not concomitant with a bleeding tendency. In such cases, the platelet count will be normal if performed on citrate anticoagulated blood.

The platelet count can also be determined in a counting chamber or estimated from a blood smear. The blood smear provides further information about the size and aspect of the platelets, about the presence of aggregates, and about potential underlying hematological disorders. Special attention should be paid to fragmentocytes (microangiopathic blood picture) since they indicate the presence of fibrin in the bloodstream and therefore the occurrence of intravascular coagulation.

**The Activated Partial Thromboplastin Time.** The activated partial thromboplastin time (aPTT) is a re-calcification time of citrate anticoagulated plasma, after preincubation with a contact activator (e.g., kaolin, silica, elaginic acid) and in the presence of phospholipid. Reference values are very much dependent on the particular reagents and should, therefore, always be specified by the laboratory.

Prolongation of the aPTT is found in cases of acquired or congenital deficiency of factors I, II, V, IX, X, XI, and XII, provided the deficiency is sufficiently severe. The coagulation factor sensitivity is dependent on the reagents used (D'Angelo et al. 1990; Naghibi et al. 1988). Deficiencies of factors VII or XIII do not affect the aPTT. The aPTT is prolonged by the presence of circulating anticoagulants and heparin. The effect of low molecular weight heparins is less pronounced. Heparin is the most common cause of a prolonged aPTT. It is uncovered by performing a reptilase time that in contrast to the thrombin time, is normal. In order to distinguish between a coagulation factor deficiency and a circulating anticoagulant, the sample is mixed 1:1 with normal plasma. In a factor deficiency, the aPTT will be normal because a half-normal concentration of a factor is more than adequate to produce an undisturbed aPTT. In the presence of a circulating anticoagulant, the aPTT remains prolonged, because even half the concentration of the inhibitor will retain a noticeable influence on the clotting time. In general, such anticoagulants are antibodies directed against phospholipids or apoprotein H (lupus anticoagulant) or against specific coagulation factors (mostly factor VIII). Fibrin degradation products may also cause a slight increase of the aPTT.

**Prothrombin Time.** The prothrombin time (PT) is the re-calcification in the presence of tissue thromboplastin. Prolongation of the PT is observed within congenital or acquired deficiencies of factors I, II, V, VII, and X. The factor sensitivity, again, is dependent on the reagent used (Naghibi et al. 1988). Most reagents cause the PT to be prolonged when the concentrations of factors I, VII, or X are 30% or less. Only a very low concentration of fibrinogen (less than 0.5 g/l) leads to a prolongation. Apart from this, the test is prolonged by high concentrations of fibrin degrada-
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tion products, by a high heparin concentration, and by some circulating anticoagulants. In general, the PT is less sensitive to inhibitors than the aPTT. The Thrombotest is a variant of the prothrombin time, in which factor V and fibrinogen are added to the reagent. This test is somewhat more sensitive than the PT for the presence of heparin and fibrin degradation products. Obviously, the Thrombotest is not sensitive to a factor V or fibrinogen deficiency. It was specifically developed for monitoring coumarin therapy. Another variant, the Normotest, is better suited for the detection of a mild deficiency of the vitamin K-dependent coagulation factors. The results of the PT are expressed either as the coagulation time measured, as the prolongation factor (measured clotting time divided by the clotting time of normal plasma), or as a percentage, indicating what dilution of normal plasma renders the same PT.

**Thrombin Time.** The thrombin time is the clotting time of citrated plasma after addition of a low concentration of thrombin. The outcome is dependent mainly on antithrombins and the coagulability of fibrinogen. A prolonged thrombin time occurs in cases of low fibrinogen concentrations (less than 1 g/L), dysfibrogenemia, and the presence of heparin (even in low concentrations), fibrin degradation products, and some paraproteins. Often, prolongation is also found with uremia and hyperbilirubinemia. It is possible to differentiate between a heparinemia and other causes by replacing thrombin with the snake venom reptilase, which is not sensitive to heparin.

**Bleeding Time.** The bleeding time is a measure for the rate of formation of a hemostatic plug in the small vessels of the skin and the vessels of the subcutaneous tissue. It is determined chiefly by the number and function of platelets and by the properties of the vessel wall. In addition, von Willebrand’s disease also causes the bleeding time to be prolonged. Standard values are very much dependent on the methodology used (Bowie and Owen 1980). The much-used technique according to Ivy is described in the following paragraph.

The principle of the bleeding time determination is the measurement of the time a standardized skin wound bleeds. The pressure on the vascular bed is controlled by a manometer cuff around the upper arm, inflated to +50 mm Hg. After disinfection, three stab wounds are made on the volar surface of the forearm with a vaccinostyle. The maximal depth of the wound that can be made with the vaccinostyle is 2 mm. Wounds should be made with a swift motion, and superficial veins must be avoided. Bleeding starts within 15 to 30 seconds. For each wound, when a drop of blood has formed, a stopwatch is started. Every 15 seconds, the blood drops are removed without touching the wound, using a round filter paper. As soon as the bleeding at a wound stops, its stopwatch is halted. The average bleeding time of the three wounds is then calculated. Reference values for this determination are, for men, up to 3 minutes and, for women, up to 4 minutes. However, the line between normal and abnormal is not absolute. A long but still-normal bleeding time by no means precludes a disorder.

Vasodilation (high room temperature, fever, drugs) may prolong the bleeding time, and vasoconstriction can mask a prolonged bleeding time. Often, in the case of an autoimmune thrombocytopenia, the bleeding time is shorter than expected on the basis of the severity of the thrombocytopenia. The opposite is true in the case of a thrombocytopenia.

**Platelet Function Disorders**

**Thrombocytopenia.** The reference range for the platelet count is 130 to 350 x 10^3/L (130 to 350 x 10^3/mm³). The number of platelets necessary for adequate hemostasis is dependent on their quality. A count of 40 x 10^3/L (40 x 10^3/mm³) will generally be associated with a virtually normal bleeding time if the platelets are functioning normally. If there is an impairment in platelet function, a count of 40 x 10^3 platelets/mm³ may lead to a large prolongation in the bleeding time. A prolonged bleeding time is always to be expected with a platelet count less than 40 x 10^3/L (40 x 10^3/mm³).

The bleeding tendency caused by thrombocytopenia shows itself in petechiae, mucosal bleeding, and hematomas. Mostly, thrombocytopenia is an acquired disorder. The causes of thrombocytopenia are listed in Table 14.2.

**Thrombocytopathy, Disturbed Platelet Function.** The combination of a prolonged bleeding time with a normal platelet count, or a discrepancy between the number of platelets and the extent of a prolongation of the bleeding time, is usually caused by a thrombocytopathy, although it may also be caused by von Willebrand’s disease. A thrombocytopathy can be either congenital or acquired. A platelet function test is necessary for the definite diagnosis of a thrombocytopathy (Day and Rao 1986). The most widely used platelet function test is the aggregation test. In one method, the degree of light scattering of platelet rich plasma is measured in a photometer. Platelet rich plasma, by virtue of its turbidity, allows little light to pass. Aggregation of platelets decreases the turbidity and therefore lets more light through. In this way, the

**Table 14.2. Causes of Thrombocytopenia**

<table>
<thead>
<tr>
<th>Disturbance in the formation of platelets</th>
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<tr>
<td>Decreased number of megakaryocytes in the bone marrow</td>
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<table>
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<tr>
<th>Disturbance in the maturation of the megakaryocytes</th>
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<td>Increased breakdown of platelets</td>
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<th>Immunological: auto- or alloantibodies</th>
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<td>Increased turnover</td>
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<tr>
<th>Damaged platelets</th>
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<tr>
<td>Loss of blood platelets: massive bleeding, transfusion of platelet-poor blood or substitution fluids, extracorporeal circuits</td>
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<th>Pooling of platelets: splenomegaly, hypersplenism</th>
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| Pseudothrombocytopenia |
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rytopathy, the platelet count and morphology are normal. Platelet function tests show no response to ADP, epinephrine, and thrombin but a normal response to ristocetin. This disorder is inherited in an autosomal-recessive fashion. It is caused by a deficiency of two glycoproteins (IIb and IIIa) from the platelet membrane (George et al. 1984). These glycoproteins make up the fibrin receptor and are essential for normal platelet aggregation. In Bernard-Soulier syndrome, another hereditary disorder, the platelet count is decreased or normal, and the platelets are very much enlarged. Aggregation is normal in response to ADP, collagen, and epinephrine but is absent in response to ristocetin. This is caused by the absence of the specific von Willebrand receptor (glycoprotein IB) from the platelet membrane (George et al. 1984). The plasma concentration and function of von Willebrand factor are normal.

In hereditary afibrinogenemia, thrombocytopenia only develops if the fibrinogen is also absent from the platelet α-granules. Other kinds of congenital thrombocytopenia are: storage pool disease (decrease of either the number of platelet-dense bodies or their content), malfunction of the release mechanism (aspirin-like defect, absent thromboxane synthesis), and the grey platelet syndrome (α-granule deficiency). Congenital storage pool disease may occur alone or in combination with other hereditary syndromes (Hemansky-Pudlak, Wisokott-Aldrich, Chediak-Higashi). A congenital release thrombocytopenia can also be found with the Marfan syndrome, the Ehlers-Danlos syndrome, mucopolysaccharidosis, and osteogenesis imperfecta.

Acquired platelet function disorders occur frequently. Most often they are caused by acetylsalicylic acid (aspirin). In such cases, the bleeding time is prolonged (the extent of the prolongation being very variable); ADP-induced aggregation is reversible; and collagen provoked aggregation is diminished or absent. Thrombocytopenia may also be caused by a significant number of other drugs. A bleeding tendency in combination with a prolonged bleeding time is often found with chronic uremia due to disturbed platelet function and also possibly to disturbed endothelial cell function. The bleeding tendency in vitamin C deficiency (scurvy) is based on the combination of an increased fragility of the vessel wall and a thrombocytopenia.

Other causes of an acquired thrombocytopenia are liver disease, myeloproliferative disorders, paraproteinemia, acute and chronic leukemia, acquired storage pool disease, acquired antibodies against platelets, thromboembolism, diffuse intravascular coagulation, extracorporeal circulation, circulating immune complexes, and vitamin B12 deficiency. Platelet function disorders are also found with congenital and acquired cardiac valve defects.

Thrombocytosis. Thrombocytosis occurs as an autonomous clinical picture (essential thrombocytosis) or in combination with other myeloproliferative disorders. A reactive thrombocytosis is found with acute or chronic inflammations, after acute bleeding, with iron deficiency, during recovery of a vitamin B12 or folic acid deficiency, with hemolytic anemia, as a paraneoplastic phenomenon, and postoperatively.

In essential thrombocytosis both bleeding (especially in the digestive tract) and thrombosis may occur. Venous thrombosis (mesentery veins, splenic vein), as well as arterial thrombosis, occur, with ischemia of the extremities, transient ischemic attacks, and amaurosis fugax. With thrombotic complications, laboratory investigation often reveals spontaneous platelet aggregation. When there is a bleeding tendency, a prolonged bleeding time and varying disturbances of platelet aggregation are often found. In general, the reactive kinds of thrombocytosis do not cause any abnormalities of platelet function tests and do not cause a bleeding tendency but, sometimes, they do induce thrombosis.

Vascular Disorders

A vasculopathy occurs as part of a number of autoimmune diseases (e.g., Henoch-Schönlein purpura, drug-induced vasculitis), with a large number of infections, with congenital vascular disorders (e.g., Rendu-Osler-Weber disease, Kasabach-Merrit syndrome), and with acquired vascular diseases (e.g. vitamin C deficiency, steroid therapy, senile purpura, cachexia). These disorders may cause a serious bleeding tendency, but laboratory tests often show no abnormalities. In particular, the bleeding time is often normal. Dysproteinemia (macroglobulinemia, multiple myeloma, cryoglobulinemia, and amyloidosis) often give rise to thrombohemorrhagic symptoms that are explained by vessel wall damage and thrombocytopenia or by impairment of platelet function caused by coating of the endothelial surface with abnormal proteins.

Coagulation Factor Deficiencies

A shortage of one or more coagulation factors, whether congenital or acquired, delays thrombin formation, with the obvious exception of afibrinogenemia. A delayed thrombin formation causes the platelet plug to be insufficiently consolidated. The consequence of this is recurrent bleeding for as many as 4 days after the trauma. Factor deficiencies may be due to a shortage of a normally functioning coagulation factor or the synthesis of molecular variants of a factor that are not functionally active.

Coagulation factors can be assayed in two entirely different ways, functionally or immunochemically. In
functional determinations, the reaction velocity is measured in a biochemical system in which the factor under investigation is rate limiting. In its simplest form the system is plasma lacking the factor in question, to which a dilution of the sample is added, and the velocity measured is the coagulation time. In this system the coagulation time is dependent on the quantity of functional coagulation factor in the sample. By comparison with dilutions of a standard plasma, the amount of the coagulation factor in the sample can be determined.

In the more recent spectrophotometric assays, chromogenic substrates are used (Witt 1991). These are oligopeptides, coupled to a chromogenic group that can be cleaved off by a particular coagulation enzyme. This gives rise to a colored product that can be measured in a spectrophotometer. Specific chromogenic substrates are available for thrombin, factor Xa, kallikrein, plasmin, and more (Table 14.1). Using purified factors, biochemical mixtures can be made in which the enzyme to be determined is rate-limiting.

Coagulation assays and chromogenic assays measure the quantity of functional coagulation factor. Immunochemical assays are fundamentally different. In these assays, the amount of antibody binding to the coagulation factor is measured. All antigenically similar molecules react with the antibody, including inactive and abnormal proteins. Therefore, molecular variants that are not functionally active will be detected by immunochemical assays.

**Hemophilia.** The hemophiliacs are inherited bleeding disorders caused by a shortage or malfunction of factor VIII (hemophilia A, 85% of cases) or factor IX (hemophilia B, 15%). Clinically, hemophilia A and B are indistinguishable. The functional concentration of the factor in plasma is usually expressed as a percentage of the concentration on plasma prepared from a pool of many healthy donors (100%). The reference ranges for factors VIII and IX are wide (50 to 150%).

It is common practice to differentiate between serious (less than 1%), moderate (1 to 5%), and mild (5 to 25%) hemophilia. The minimal concentration for safely carrying out surgical operations is greater than 40% for factor VIII and greater than 30% for factor IX. This means that there are patients having plasma concentrations between 25 and 40% who do not have a bleeding tendency under normal circumstances but who do have a significant chance of (recurrent) bleeding during or following operations that demand much from hemostasis (tonsillectomy, dental extractions). Since the concentration of factor VIII increases under the influence of stress or anemia, patients with mild hemophilia A may show low-normal values of factor VIII, which may create rather difficult diagnostic problems.

The aPTT may be normal in mild hemophilia; in moderate or serious hemophilia it is generally prolonged. The diagnosis is confirmed using a specific coagulation factor measurement.

Antibodies may arise as a complication of the substitution therapy of hemophilia. For the determination of the antibody titer a standardized method has been developed (Bethesda test). Subject to the level of the titer, patients are designated as low or high responders.

Both hemophilia A and B have X-linked heredity. Therefore, it is important to determine carriership in the female relatives of hemophilia patients. Carriers of a hemophilia gene have a factor concentration of 50% ± 25%. Some have a low coagulation factor concentration (<25%) with concomitant bleeding tendency. Others have a concentration of greater than 75% and are therefore difficult to identify as a carrier. It is possible to use clinical data to calculate the chances of someone being a carrier, but it is much more reliable to determine carriership by way of DNA analysis (White and Shoemaker 1989; Thompson 1986).

**von Willebrand's disease.** Patients with von Willebrand's disease have a congenital shortage or an abnormal kind of von Willebrand factor. von Willebrand factor is a large plasma protein, circulating as a multimeric protein complex consisting of 2 to 20 molecules. von Willebrand factor has two functions. In the first place, it is necessary for the adhesion of platelets to the subendothelium. A disturbance of this function shows in vitro as a prolonged bleeding time, and in vitro as decreased platelet aggregation in response to ristocetin. In the second place, it serves as a transport protein for factor VIII. A shortage of von Willebrand factor is thus accompanied by a shortage of factor VIII with clinical and laboratory findings consistent with the degree of factor VIII deficiency.

On the basis of the inheritance pattern and the laboratory findings, a distinction is made between a number of different types of von Willebrand's disease (Tuddenham 1989). Type I is the classical variety, caused by a reduced concentration of otherwise normal von Willebrand factor. Type II is a molecular variant with an abnormal multimeric pattern. The multimeric pattern can be evaluated by electrophoretic techniques. By reason of mutual differences in this abnormal multimeric pattern, several subtypes are discerned. The concentration of von Willebrand factor and its functional activity are usually slightly decreased but often not to the same degree. Type IIb is of particular clinical importance, as platelet function tests show an increased sensitivity for ristocetin and, sometimes, for thrombocytopenia and megathrombocytes. The factor VIII binding capacity may be normal so that its concentration is not necessarily lowered. Type IIb has to be differentiated from pseudo-von Willebrand's disease or the so-called platelet type. This disorder is caused by abnormal platelets having an increased affinity for normal von Willebrand factor.

**Other Hereditary Coagulation Disorders.** Congenital afibrogenemia is a very rare disorder, noticeable because plasma is incoagulable in all tests. Some families with the disorder have a serious bleeding tendency and a high mortality because of cerebral hemorrhage shortly after birth. In other families clinical symptoms are very mild or lacking altogether. This difference is probably caused by the fact that some-
times only plasmin fibrinogen is absent (mild disease), whereas in other cases platelet fibrinogen is also deficient. The latter case results in platelet function disturbances (prolonged bleeding time) because fibrinogen adherence is necessary for a normal platelet function.

Congenital hypofibrinogenemia occurs in several patterns of inheritance and usually has little clinical significance. Female patients sometimes have an increased risk of habitual abortion and bleeding during parturition. Congenital dysfibrinogenemia in general does not induce an increased bleeding tendency, but it is sometimes associated with a thrombotic tendency and disturbed wound healing. The first laboratory indication of an abnormal fibrinogen molecule is an unexplained prolonged thrombin time. Often, the repletion time is prolonged as well. Also, there is a discrepancy between the functional fibrinogen determination and immunological assays. The diagnosis can be confirmed by investigation of the purified fibrinogen or by detection of a genetic polymorphism.

Deficiencies of factor XII and prekallikrein do not cause a bleeding tendency. The cases of factor XII deficiency, accompanied by a thrombotic tendency, are explained by a diminished activation of the fibrinolytic system. Several such cases have been reported. On the other hand, several cases of severe factor XII deficiency have been described in families with no thrombotic tendency at all. A prekallikrein deficiency mildly prolongs the aPTT in heterozygotes. This prolongation shortens upon longer incubation with the contact activator because the defect is corrected by activation of factor XII in vitro.

Homozygous factor XIII deficiency causes a bleeding tendency of varying severity and a disturbance of wound healing, often with keloid scar formation. All sorts of bleeding can be found. There is even a risk of a high-mortality cerebral hemorrhage just after birth. A classical indication for a factor XIII deficiency is bleeding on detachment of the umbilical cord. All global coagulation tests are normal. The diagnosis is made by demonstrating that clotted blood dissolves in a 5% urea solution. Note that this test is only positive when factor XIII concentrations are below 5%. False-positive results may ensue in diffuse intravascular coagulation (DIC) and in the presence of paraproteins. The diagnosis of a factor XIII deficiency can be confirmed, using immunological techniques.

**Acquired Coagulation Disorders.** Acquired coagulation disorders arise as a consequence of disturbed production (vitamin K deficiency, vitamin K antagonists, liver disorders), increased turnover (diffuse intravascular coagulation), and increased loss of coagulation factors (massive bleeding, disorders accompanied by protein loss like the nephrotic syndrome). A rare cause is neutralization of specific coagulation factors by antibodies, referred to as circulating anticoagulants. They may occur spontaneously or arise after substitution therapy (in hemophilia A, 5 to 10% of cases). Spontaneously appearing antibodies may arise during and after pregnancy, with asthma, eczema, ulcerative colitis, chronic glomerulonephritis, systemic lupus erythematosus (SLE), malignancies of the lymphatic apparatus, other disorders of the immune system, and as a side effect of drug therapy (e.g., penicillin, sulfonamide). Apart from circulating anticoagulants against specific coagulation factors, antiphospholipid antibodies occur as well (Tripplett 1990). It has recently become clear that the "lupus anticoagulant" is often directed against apoprotein H, a protein that itself attaches to phospholipid. The use of washed platelets (which are apoprotein H-free), rather than cephalin, as the source of phospholipids in the aPTT leads to normalization of the test results. Clinically, the presence of lupus anticoagulants is not associated with a bleeding tendency but, for reasons yet to be clarified, with habitual abortion and arterial and venous thrombosis. A bleeding tendency only occurs in those cases in which the circulating anticoagulants are antibodies against individual coagulation factors or blood platelets.

Acquired coagulation disorders seen in paraproteinemia and amyloidosis arise by absorption of specific factors (factor V, factor X) to the abnormal proteins or by interference with the action of thrombin, with the polymerization of fibrin monomers, or with procoagulant phospholipids.

**Vitamin K Deficiency.** A shortage of vitamin K (either an absolute shortage due to inadequate intake or a relative shortage due to the use of vitamin K antagonists) results in a decrease in the concentrations of the functional forms of vitamin K dependent coagulation factors II, VII, IX, and X and the anticoagulant factors protein C and protein S. The factors in question are still being produced by the liver and also partly secreted into the blood stream, but they are inactive because they lack the γ-carboxyglutamic acid side chains, which are necessary for the function of these proteins. They are known as PIVKAs, (proteins induced by vitamin K absence), or decarboxyfactors. The PIVKAs are weak inhibitors of coagulation (Hemker et al. 1963).

In the laboratory, a prolonged prothrombin time and aPTT are found. Thrombotest and Normotest have a specific sensitivity for vitamin K shortage, because they contain added factor V and fibrinogen. These tests are not sensitive to an isolated deficiency of factor IX, but they are for the inhibiting action of structurally abnormal factor IX molecules. In order to prove a vitamin K deficiency, it is essential to demonstrate the presence of decarboxyfactors. It is possible to measure the concentrations of carboxylated and noncarboxylated prothrombin together by using immunological techniques or nonphysiological activators (Echis carinatus poison, staphylococaglase). If these techniques reveal a factor II concentration greater than the functional factor II concentration, the presence of PIVKAs is probable. The most reliable method for demonstrating small quantities of noncarboxylated factors is based on the use of specific monoclonal antibodies. This, as well as a direct determination of the
vitamin K concentration by liquid chromatography, is only carried out in specialized laboratories.

**Diffuse Intravascular Coagulation.** It still is a matter of debate whether a slight degree of thrombin generation in vitro is a normal phenomenon or not. Using the sensitive methods available today, it is often possible to demonstrate low plasma concentrations of fibrinopeptide A, fibrin(ogen) degradation products, and thrombin-antithrombin III complexes without the existence of a clear underlying pathology. Somewhat higher concentrations of these products can be found in a variety of diseases, e.g., liver insufficiency, acidosis, hypoxemia, or hypotension. If the products are present in concentrations above a certain, rather arbitrary limit, one speaks of DIC (Bick 1988, Müller-Berghaus 1989). If the process is not too severe, an increase of the products of the coagulation process is found without a significant decrease in the coagulation factors and platelet counts. With uncompensated DIC a paradoxical situation arises. Circulating microthrombi (platelet aggregates and/or fibrin precipitates), on the one hand, give rise to microembolization and thereby to organ damage. On the other hand, a generalized bleeding tendency ensues. This bleeding tendency is caused by an insufficiently compensated consumption of coagulation factors and blood platelets and by a secondary increase of fibrinolytic activity. Moreover, the degradation products liberated by this process interfere with platelet function and fibrin polymerization. Furthermore, an increased consumption of α2-antiplasmin may result in the occurrence of free plasmin, which specifically inactivates fibrinogen and the coagulation factors V and VIII.

In practice, the determination of fibrinopeptide A and thrombin-antithrombin III complexes has been found of little use in the laboratory evaluation of DIC because the assays for these products are very time consuming. Fibrin monomers can be demonstrated rapidly by way of the ethanol gelation test or using one of the more sensitive fibrin monomer immunnoassays presently commercially available (Bick 1988). Also, new techniques based on the use of monoclonal antibodies have been developed that enable the determination of degradation products of fibrinogen separate from fibrin degradation products. In heparinized patients (or if the blood sample is taken from heparinized catheters), the fibrin degradation product (FPD) determination may be falsely positive. The laboratory analysis of DIC should also include an evaluation of a peripheral blood smear. In about half of all cases, the smear will show a microangiopathic blood picture with fragmented nuclei. The laboratory analysis only gives a "snap-shot" of the dynamic processes in DIC, so it should be repeated regularly.

Serious liver disorders are often difficult to differentiate from DIC on the basis of laboratory tests. The presence of a clear microangiopathic picture favors DIC. Other disorders from which DIC needs to be differentiated are primary lysis of fibrinogen (in which there is generally a normal platelet count and antithrombin III concentration), thrombotic thrombocytopenic purpura (in which there is usually a normal fibrinogen concentration and a normal antithrombin III concentration, and little consumption of coagulation factors), and the hemolytic uremic syndrome (which very much resembles thrombotic thrombocytopenic purpura diagnostically). With thrombotic thrombocytopenic purpura and hemolytic uremic syndrome, in particular, the microangiopathic blood picture and disseminated microthrombi (platelet aggregates) are prominent.

**Liver Disorders.** Loss of hepatic parenchyma leads to a diminished synthesis of plasma proteins and thereby to decreased concentrations of the coagulation factors which, with the exception of von Willebrand factor, are produced by the liver. Given the short half-life of factor VII, its concentration is a useful measure of acute changes in the protein synthesis capacity of the liver. In practice, determination of the PT or use of the Normotest are most suitable. In acute hepatitis, the aPTT is less appropriate because the concentration of factor VIII is often elevated. The average concentration of the vitamin K dependent coagulation factors has prognostic significance, if vitamin K deficiency can be precluded. If the average concentration is not too much below 25%, the prognosis is moderately favorable; if it is less than 10% it is unfavorable, there being a mortality of almost 100%. The concentration of factor V also has a prognostic value, but it should be remembered that the production of factor V is stimulated by cholestasis, which usually is present in acute hepatitis. The prognosis is very unfavorable if the factor V concentration is below 10%. Apart from a decreased synthesis, the following disorders can also contribute to the bleeding tendency in liver disease: DIC, thrombocytopenia, thrombocytopeny, increased fibrinolytic activity (due to insufficient clearance of plasminogen activator, reactive release of tissue plasminogen activator (t-PA), or insufficient production of α2-antiplasmin), and disturbed fibrin formation. The thrombocytopenia with liver insufficiency is based on hypersplenism, consumption caused by DIC, and/or a production defect (e.g., with alcohol abuse or folic acid deficiency).

**Disorders of Hemostasis Caused by Massive Blood Transfusions.** A serious bleeding tendency may arise in grave, acute blood loss, where the entire blood volume needs to be replaced by way of transfusions within 24 hours. This is caused by a deficiency of coagulation factors and thrombocytes. In a number of cases, a thrombopathy may also occur as a consequence of the use of plasma substitutes in the initial phase of blood volume support. Regular control of the PT, the aPTT, and the platelet count are indicated. In cases in which there is a suspicion of DIC, it is useful to determine the thrombin time and the fibrinogen concentration as well. If DIC is occurring, a prolongation of the thrombin time and a decrease of the fibrinogen concentration will be seen. DIC may even be the cause of the bleeding. Massive transfusion, on the other hand, is able to provoke DIC to a certain extent.
Disorders of Hemostasis in Cardiopulmonary Surgery Involving Extracorporeal Circulation. Cardiopulmonary surgery with extracorporeal circulation is always accompanied by abnormal hemostasis tests and sometimes with serious bleeding. The most common abnormalities are thrombocytopenia, thrombocytopenia, coagulation factor deficiencies, DIC, primary fibrinolysis, and heparin-induced problems, or a combination of these. Bleeding is, in the majority of patients, determined by a platelet function disorder and an increase in fibrinolysis. The administration of aprotinin, an inhibitor of proteolytic enzymes, during heart surgery results in a considerable reduction in postoperative bleeding and in the transfusion requirement. This favorable effect is ascribed to the inhibition of plasmin. Free plasmin also appears to be able to cause an adhesion defect of platelets by affecting glycoprotein Ib. Heparin-induced problems may be associated with the administered heparin and its neutralization by protamine. Too little, as well as too much protamine, leads to coagulation disorders. A heparin "rebound" may ensue by dissociation of the heparin-protamine complex. This effect occurs less with protamine sulphate.

Increased Breakdown of Fibrin. A bleeding tendency caused by increased fibrinolysis may be the consequence of a high activity of plasminogen activator or a low activity of physiological inhibitors. Sometimes the substratum (fibrin) is abnormal. Congenital disorders of increased fibrinolysis include α2-antiplasmin deficiency (an abnormal α2-antiplasmin molecule is present, resulting in lowered functional concentrations but normal antigen concentrations), abnormal plasminogen activator inhibitor-1, and several kinds of dysfibrinogenemia. Acquired disorders include DIC with secondary fibrinolysis (increased concentrations of fibrin- and fibrinogen degradation products, short euglobulin clot lysis time, and increased t-PA activity), primary fibrinogenolysis, e.g., with certain kinds of leukemia (low concentrations of fibrinogen, high concentrations of fibrinogen degradation products, and high u-PA and t-PA activities), and thrombolytic therapy (low-fibrinogen concentrations, high fibrinogen degradation products, high t-PA activity, and low α2-antiplasmin concentration).

Bleeding Tendency with No Abnormalities in Routine Laboratory Studies. The medical history and physical examination may indicate a clear bleeding tendency, while the routine laboratory studies disclose no abnormalities. This situation is usually found in varieties of von Willebrand's disease, hemophilia, or thrombocytopenia. Stress, e.g., anesthesia, and other negative reactions may temporarily mask such defects. Investigation of platelet function and determination of the factor VIII concentration (in rare cases, other factors) sometimes even reveal a moderately serious disorder. The bleeding tendency may also be caused by an accelerated breakdown of fibrin, a factor XIII deficiency, or vascular disorders. With vascular disorders, routine hemostatic studies are often completely normal despite the sometimes impressive bleeding tendency.

Finally, it should be noted that a diminished prothrombin consumption is often the only abnormal laboratory result found in mild bleeding tendencies that are not further classifiable.

THROMBOTIC TENDENCY

Laboratory investigation of a thrombotic tendency is indicated in patients having unexplained thrombosis at a young age (less than 45 years old) and a positive family history. Only in about one third of the cases will an abnormality be found that explains the thrombotic tendency.

Antithrombin III deficiency is transmitted in an autosomal dominant fashion. Absolute antithrombin III deficiency (homozygous disease) does not occur, probably because it is incompatible with life. Partial deficiencies (heterozygous disease) usually show a concentration around 50%. The most frequent type, type I, has both a lowered functional concentration and a lowered antigen concentration. Structural variants of the antithrombin III molecule also exist (type II) (Tollefson 1990). Some show a normal antigen concentration but a decreased functional concentration, and others show a normal antithrombin III concentration but cannot be stimulated by heparin. When a decreased antithrombin III concentration has been ascertained, it should be determined whether it is an acquired deficiency resulting from decreased production (liver disease, asparaginase therapy, estrogen therapy), increased turnover (serious bleeding, diffuse intravascular coagulation, heparin therapy, recent thromboembolism), or loss via urine (nephrotic syndrome).

Congenital deficiency of protein C or S is usually inherited in an autosomal dominant fashion. In the Netherlands, the prevalence of protein C deficiency in a population of nonrelated families with thrombophilia in the Netherlands is about 5 to 10%. In addition to the dominant form, an autosomal recessive protein C deficiency exists. Shortly after birth, homozygotes develop the picture of a neonatal purpura fulminans. In these families, heterozygotes seldom suffer from thrombosis. Therefore, not all individuals with a protein C deficiency have an increased risk of thrombosis. Similar to antithrombin III deficiency, two types of protein C deficiency are distinguished: type I (low functional concentration, low concentration of antigen) and type II (low functional concentration, normal concentration of antigen) (Miletich 1990). As yet, there is no consensus about the classification of protein S deficiency. This is, among other things, the consequence of not yet having elucidated the role of the C4-binding protein, which binds part of the protein S present in plasma. One useful classification is the following: patients with a decreased total protein S and a decreased free protein S antigen (not bound to the C4-binding protein) and patients with a normal total but a lowered free protein S antigen. Functional
protein S assays are being developed (Comp 1990). For the interpretation of laboratory results, it is important to recognize that treatment with coumarin derivatives and vitamin K deficiency also lead to protein C and S deficiency.

Congenital disorders of fibrinolysis may also be accompanied by a thrombotic tendency. This is known to be true for certain types of congenital dysfibrogenemia, and hypoplasminogenemia. Families have been described, in which a connection appeared to exist between a thrombotic tendency and an elevated concentration of histidine-rich glycoprotein, a protein interfering with the binding of plasminogen and t-PA to fibrin. Acquired disorders of fibrinolysis probably are fairly common, although the precise clinical importance of these disorders is not clear. Increased concentrations of α2-antiplasmin, increased plasminogen activator inhibitor concentration, and disturbances in the production or release of t-PA can be found (Bachmann 1990). The latter aspect can be examined by determination of t-PA antigen and plasminogen activator inhibitor concentration before and after stimulation (venous occlusion, desmopressin acetate (DDAVP)). Long-term treatment with inhibitors of fibrinolysis (e-aminocaproic acid, cyklokapron, translyi) is associated with an increased risk of thrombosis.

An acquired thrombotic tendency also occurs in patients who have lupus anticoagulants. The lupus anticoagulant is associated with arterial and venous thromboembolism, habitual abortion, and thrombocytopenia. A causal relationship between the presence of this anticoagulant and the occurrence of thrombosis has not been established, however.

Thrombosis often develops in the myeloproliferative disorders, especially in polycythemia rubra vera with or without thrombocytopathy. Platelets may be abnormal, and thrombocytopathy is a common finding. The presence of this anticoagulant and the occurrence of thrombosis has not been established, however.

Oral Anticoagulation

The effect of oral anticoagulation is measured using the PT or one of its variants (Hirsh 1991). It is sensitive to changes in the concentrations of factors I, II, V, VII, and X. With oral anticoagulation, the concentrations of factors II, VII, IX, and X vary. In order to eliminate the test's sensitivity to changes in the concentrations of factors I and V, which are not relevant in this case, a reagent is often used that contains these factors (e.g., Thrombotest). The result of the PT is a clotting time. In order to appreciate the meaning of this value, it can be compared to the clotting time of normal plasma. As another possibility, normal plasma can be diluted, and the clotting time of each of the dilutions can be measured. In this way, it can be established which dilution of normal plasma produces a clotting time equal to that of the patient's plasma. The result is represented as a percentage (e.g., a dilution of 1:5 is 20%). The main problem is that different thromboplastins have different sensitivities for a decrease in the concentrations of factors II, VII, IX, and X, as well as for the inhibition caused by noncarboxylated factors (PIVKAs). This causes the same degree of anticoagulation measured with different thromboplastins, to give rise to different prothrombin times. Comparison with an international standard thromboplastin and application of a specific calculation have resulted in the so-called international normalized ratio (INR). This is the ratio of the PT of the sample to the PT of normal plasma, corrected for the variation of the particular thromboplastin. Application of the INR allows the range of an adequate oral anticoagulation to be known, independent of the thromboplastin used.

Still, the PT is not the ultimate test for monitoring oral anticoagulation, since the same result may be produced by all sorts of variations between the three coagulation factors involved (Xie et al. 1989b). At the beginning of a therapy, the factor VII concentration (half-life, 6 hours) decreases much faster than the factor II concentration (half-life, 60 hours). Likewise, after administration of vitamin K, factor VII concentrations are back to normal much sooner. If, e.g., a prothrombin time of 18 seconds is found at the beginning of a treatment, the implications are quite different, as compared to the same value found during a long-term treatment or after administering vitamin K. Furthermore, every thromboplastin is sensitive to a different degree to variation in the concentrations of factors II, VII, and X. The thrombotic or bleeding tendency of a patient is also dependent on these, but in another completely unknown way. This may lead to long debates about the usefulness of the factor sensitivity of a thromboplastin. In practice, every thrombo-
plastin is satisfactory, provided that its therapeutic range comprises a reasonable number of seconds, so that deviations of 1 or 2 seconds do not have an important influence on the outcome.

Oral anticoagulant therapy, monitored using the PT, calls for experience with the anticoagulant drug, as well as with the method of control. In the Netherlands, monitoring is usually taken care of by the specialized thrombosis care centers. It should be clear from what has been stated above, though, that truly rational control of anticoagulation will not be possible until research has shown which changes brought about by therapy are responsible for the therapeutic effect and to what degree.

Heparin

The first question to be asked regarding the control of heparin therapy is whether the effect of heparin should be controlled at all. With prophylactic doses, control is generally not necessary. In heparin therapy for extracorporeal circulation, it will always be required. Views differ on its purpose in case of therapeutic dosages. This is due in part to the fact that, although the results of a number of tests are known to vary in response to the administration of heparin, it is not known whether, or to what extent, they reflect the therapeutic effect. The most important determinations available are the aPTT, the thrombin time, the antithrombin activity, and the antifactor Xa activity.

For the determination of the antithrombin and the anti-Xa activity, a fixed amount of thrombin or factor Xa is added to the patient's plasma sample, and the amount remaining after a fixed incubation time is measured. The rate of inactivation of these factors is dependent on the amount of antithrombin III in the sample and the quantity and nature of the heparin present. Some test kits are designed such that they are independent of the antithrombin III concentration of the sample. (These kits measure the amount of heparin in the sample but do not indicate whether it can exert its action.) It has been put forward that the antifactor Xa activity correlates with heparin's antithrombotic effect and the antithrombin activity with the bleeding tendency caused by heparin treatment. However, the antifactor Xa activity hardly influences thrombin formation and thus is very unlikely to contribute to the effect of heparin in vitro. It is, though, a helpful side effect of heparin therapy since it can be measured rather sensitively allowing, in particular, the concentration of low molecular weight heparins to be determined fairly well. It should be remembered, however, that the relation between antifactor Xa activity and biological effect may be distinct for different kinds of heparin. The antithrombin activity is less sensitive and is used less often, although it probably is more relevant pathophysiologicaly. Both determinations are in general carried out, using bovine coagulation factors and in the absence of Ca$^{2+}$, whereas in vitro, human coagulation factors are inactivated in the absence of Ca$^{2+}$. The differences between these situations may have significant effects on the measurement of different kinds of heparin.

The thrombin time is insufficiently accurate and sensitive to detect the low concentrations of heparin present during prophylactic heparin therapy. It is, on the other hand, a useful indicator in the titration methods used for assessing the high concentrations of heparin present during hemodialysis and heart surgery involving cardiopulmonary bypass. In the titration methods, the heparin present in the sample is neutralized by known amounts of protamine to find the concentration that just nullifies the heparin effect.

The most useful heparin determination is the aPTT, provided that no extreme accuracy is required. The effect of heparin on the test is largely due to the inhibition of the thrombin-mediated activation of factor VIII. In addition, the inhibition of factor IXa under the influence of heparin plays a role. The aPTT is less sensitive to low molecular weight heparins but still remains a useful tool.

It is very important to realize that even a slight activation of blood platelets in the sample may considerably reduce the amount of available heparin, owing to release of heparin binding substances (platelet factor 4) by the platelets. An accurate heparin determination thus requires blood to be taken in a tube containing an inhibitor of in vitro activation of platelets.

In summary it can be stated that the ideal test for controlling the effect of heparin does not exist. For clinical purposes it can be said that: no control is necessary for prophylaxis, the aPTT is a sufficient tool for monitoring the treatment with unfractionated (normal) heparin, the antifactor Xa test is acceptable for controlling therapy with low molecular weight heparins, and the thrombin time is useful for heparin neutralization tests. In cases of bleeding in individuals receiving heparin therapy, the platelet count and fibrinogen concentration should also be determined in order to preclude a heparin-induced thrombocytopenia or DIC.

Thrombolytic Therapy

In thrombolytic therapy, a number of highly abnormal laboratory results are found, irrespective of the thrombolytic drug used (Marder and Sherry 1988). Fibrinolytic activity can be determined by way of either the euglobulin clot lysis time, the t-PA activity, or the free plasmin concentration. In practice, the usefulness of these tests is minor.

The consumption of coagulation factors is measured by the determination of fibrinogen, plasminogen, α2-antiplasmin, and factors V and VIII. The presence of reaction products is assessed by the determination of fibrin- and fibrinogen degradation products. Functional fibrinogen determination based on thrombin-mediated clotting is not reliable because of the interference of high titers of fibrinogen degradation products. Because of this, a very low concentra-
tion of fibrinogen is often found in the first hours after the onset of treatment. An immunological assay that is not disturbed by FDPs or a turbidimetric fibrinogen determination renders more reliable data.

A potential problem in the evaluation of thrombolytic therapy is that the proteolytic activity may continue in the specimen container, causing the test results not to be a reflection of the situation in vitro. This can be precluded by having inhibitors of fibrinolysis (trianexamic acid, e-aminocaproic acid, aprotinin).

The predictive value of the laboratory results for the risk of bleeding and/or reocclusion is low, which means that, in practice, laboratory control is not very useful. It is usually sufficient to demonstrate the lytic state once by documenting a large reduction of the fibrinogen concentration and to monitor the aPTT, in particular to establish a starting-point for subsequent heparin therapy. The aPTT is generally not prolonged very much.

EXERCISES

14.1. What laboratory studies should be ordered in the evaluation of a patient who complains of the recent onset of recurrent nose bleeds, bleeding gums, and scattered petechiae?

14.2. Not infrequently, patients with cancer experience disordered hemostasis. Describe some of the mechanisms by which cancer and anticancer therapy can result in disordered hemostasis.

14.3. A patient who has been receiving warfarin for 2 days has a PT of 18 seconds (control, 12 seconds; PT ratio, 1.5). For the thromboplastin used, the INR (international normalized PT ratio) is 2.0. The patient has a calf vein thrombosis for which the recommended INR intensity is 2.0 to 3.0. Should this patient’s warfarin dose be changed?

REFERENCES


