Thrombin generation assays

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Thrombin generation assays: accruing clinical relevance
H. Coenraad Hemker, Raed Al Dieri and Suzette Béguin

Purpose of review
After decades of near oblivion, thrombin generation is being revived as an overall function test of the plasmatic coagulation system in platelet-poor plasma (PPP). In platelet-rich plasma (PRP) it assesses platelet procoagulant functions as well.

Recent findings
The recently developed use of special fluorogenic thrombin substrates allows monitoring of thrombin concentration in clotting PPP and PRP on line in up to 24 parallel samples. Studies in model systems stress the importance of cell-bound thrombin generation such as measured in PRP.

Summary
The method can be profitably applied to various hitherto unyielding problems such as the control of (low-molecular-weight) heparin therapy, the detection of lupus anticoagulant, and various forms of thrombomodulin and activated protein C resistance (including the use of oral contraceptives) as well as monitoring the treatment of hemophilia by factor VIII bypassing therapy. In PRP it reflects the abnormalities encountered in von Willebrand disease and Glanzmann and Bernard-Soulier thrombopathy as well as the action of antiplatelet drugs.

Keywords
thrombin generation, platelet-poor and platelet-rich plasma, monitoring antithrombotics, lupus anticoagulant, hemophilia and von Willebrand disease

Introduction
Because of its numerous positive and negative feedback controls, the hemostatic-thrombotic system is so complicated that it is practically impossible to judge the overall hemostatic function of the blood from the concentration or structure of its components. Information on details of the system therefore is not an alternative for an overall function test. Clotting times (prothrombin time, activated partial prothrombin time [aPTT], activated whole blood clotting time) do not indicate hypercoagulability and are insensitive to mild bleeding disorders. For over a century (eg, Hayem [1]), the generation of thrombin in clotting blood or plasma has been used to assess the coagulation system, but only recently have technical developments brought it into reach of the nonspecialized laboratory.

Ex vivo thrombin generation (TG) should be distinguished from in vico TG, revealed by products of an ongoing clotting process in the body (prothrombin fragment 1-2, d-dimer etc.). Increased in vico TG indicates an ongoing pathologic process. Increased or decreased ex vivo TG means that the function of the coagulation process is abnormal (eg, hyperprothrombinemia [2], hemophilia [3••], use of anticoagulants [4]). It does not necessarily mean ongoing pathology but indicates an increased risk of thrombosis or bleeding [5]. In vico TG is a smoke detector signaling ongoing evil; ex vivo TG is like the smell of gasoline indicating an increased risk.

The first law of hemostasis and thrombosis
“The more thrombin the less bleeding but the more thrombosis, the less thrombin the less thrombosis but the more bleeding” may be called the “first law of hemostasis and thrombosis.” The converse is not true. The cause of thrombosis or bleeding can be in the vessel wall, with the hemostatic function of the blood being perfectly normal. The circumstantial evidence for the first law is overwhelming; we know of no refutation. Bleeding may be caused by a lack of any known clotting factor or by an excess of antithrombin activity (antithrombin Baltimore, heparin); thrombosis by an excess of clotting factor (eg, prothrombin); or a shortage of coagulation delimiters (antithrombin, proteins C and S).

The time course of TG (the “thrombogram”) is shown in Figure 1. After a lag time, a burst of thrombin is observed. Clotting occurs at the end of the lag time, when more than 95% of all thrombin is still to be formed. We
may well ask what the purpose is of all the thrombin that is formed after clotting [6]. We see two main functions: In vivo thrombin diffuses out, around the primary focus of its formation (e.g., wound, ruptured plaque). Above a certain threshold concentration, it will autocatalytically promote more thrombin formation and thus thrombus growth. Under that threshold it will be washed away or neutralized. The amount of thrombin formed in a focus will thus determine the extent of a thrombus/hemostatic plug. Thrombin in a clot also prevents subsequent isviathe activation of thrombin-activatable fibrinolysis inhibitor [7,8]. This explains why hemophilic bleeding often develops after a bleeding-free interval, as if a formed hemostatic plug is precociously dissolved (e.g., Verstraete [9]). Thrombin also acts on a number of different cells in the neighborhood of a focus and has a function in tissue repair and proliferation of surrounding cells [10,11].

Models of ex vivo thrombin generation
The course of thrombin concentration in a hemostatic plug or thrombus is technically impossible to measure. Measuring thrombin (-products) in samples from the blood in a wound comes close [12,13], as does subsampling from clotting blood [14,15]. Both require heavy experimentation. Two essential different types of model are of more practical use: reconstituted systems and plasma.

Reconstituted systems (for reviews see Monroe et al. [16] and Mann [17•]) use purified clotting factors to represent the physiologic situation. Reaction conditions are under tight control and can be varied at will. To investigate TG at cell surfaces, notably platelets with or without cells bearing tissue factor (TF) or thrombomodulin (TM) (monocytes, endothelial cells) they proved very useful [18]. However, purified factors will not necessarily retain their native activity (e.g., Hemker [19]). Reconstituted systems are as realistic as our insight into the clotting mechanism allows; extrapolation to physiology should therefore be regarded with due suspicion. Minor players (e.g., β1 integrin, α2macroglobulin [αM]) and a fortiorti unrecognized proteins/functions escape notice; fibrin(-ogen) and von Willebrand factor (vWF) are often absent but do play a role in rendering platelets procoagulant [20]; see below).

Thrombin generation in plasma (in platelet-poor or platelet-rich plasma, PPP or PRP [21,22]) represents the function of a relevant slice of the in vivo system with all the plasma proteins present, unmodified, near their physiologic concentrations and independent of a priori hypotheses. It represents a function test of the “isolated organ” PPP or PRP. The vessel wall is lacking, however. To simulate its presence, the two most important known elements, TF and TM, may be added to the plasma (Fig.1).

Cell-bound thrombin generation: the role of platelets
The arm-to-tongue circulation time of the blood (~30 seconds) is short compared with a whole blood clotting time, so thrombin formed in flowing blood in vivo is rapidly diluted and inactivated before clotting can occur. Thrombin will only build up in unstirred boundary layers at cell surfaces and in the unstirred plasma caught in a clot or an aggregate. Transport by diffusion will therefore tend to govern reaction rates. According to our interpretation [23] diffusion limitation, for instance, explains the kinetics observed in a cell-bound model of TG by Allen et al. [24•].

Cell-bound TG is dependent on TF-bearing cells (monocytes, perivascular cells) and platelets [18,16]. The role of the platelet in physiologic thrombin generation is twofold. By adhesion and aggregation it forms a maze in which plasma can clot without the thrombin being washed away; conversely, activated platelets provide the surface on which TG can take place. Upon activation of the platelet [25], procoagulant phospholipids appear at its outside. Thrombin (PAR 1) and collagen (GPVI) bring about this process, especially in combination. GPIIb/IIIa plays a role as well and GPIIb/IIIa antagonists inhibit [26]. vWF adsorbs onto polymerizing fibrin and this probably brings about a molecular change (like ristocetin), which makes it interact with GPIIb-V. This enhances the formation of a procoagulant surface [27,28].

Techniques of thrombin generation measurement
The thrombogram can be obtained through subsampling [29] or through monitoring the conversion of a suitable substrate directly added to the clotting plasma [30]. The former method is straightforward and time consuming;
the latter allows automatic continuous measurement of many samples in parallel. Via subsampling, the thrombin (∼30% of total) that adsorbs onto the formed clot [31,32,33] escapes notice. Such thrombin can activate factors V, VIII, and XI or platelets and thus probably is essential in thrombin growth [34].

Added thrombin substrate occupies part of the thrombin formed. Enough free thrombin should remain to allow for natural feedback reactions and for adequate removal of thrombin by antithrombins; therefore binding should be relatively loose (low Km). Suitable substrates should also be converted slowly (low k_{cat}) so as not to be consumed during the experiment [35].

The fluorescent signal has the drawback of not being linear with product concentration. To compensate for this and for the effects of substrate consumption, the calibrated automated thrombogram (CAT) method has been developed that continuously compares the signal from the experimental sample to that of a fixed known thrombin activity [36•]. This method allows visualizing the thrombin concentration in clotting PPP or PRP in 24 parallel experiments.

The thrombogram

Typical thrombograms as obtained with the CAT are shown in Figure 1. The three most important parameters are the lag time, the peak value, and the area under the curve or endogenous thrombin potential (ETP), which quantifies the enzymatic “work” that thrombin can do during its lifetime (“person-hours” of thrombin) [37]. Plasma clots at the end of the lag phase so the clotting time can be read from the thrombogram. During the lag phase the reaction mechanisms are essentially different from those during the thrombin burst [15]; this is one of the reasons that the clotting time does not represent TG. The normal values and coefficients of variation as obtained with the CAT-method are given in Table 1.

Partial techniques

Both the lag time and the ETP can be obtained by alternative techniques without monitoring the complete thrombogram. The clotting time represents the lag time. The ETP can be assessed by measuring the product from any natural or added substrate that is not exhausted during the clotting process. One natural substrate is α2M, which, in defibrinated plasma, will bind ∼30% of the thrombin formed (∼5% with fibrinogen). The final concentration of the (amidolytically active) α2M-thrombin complex is proportional to the ETP [30]. Rosing et al. [38] used this approach to demonstrate acquired activated protein C (APC) resistance through the use of oral contraceptives. The ETP can also be assessed by measuring the end level of conversion of a slow-reacting artificial substrate, provided that it does not react with α2M-thrombin.

Surrogate techniques of thrombin generation

Several techniques have been published that depend on fibrinogen polymerization. Apart from the clotting time, they give little information on thrombin generation because fibrinogen is exhausted before 5% of all thrombin is formed. In so far as the properties of the clot are determined by the velocity or the amount of thrombin formed, some information can be retained, however. Indeed tensile strength [39•], clot retraction [40•], turbidity [41], and fibrinolysis [42•] are derived variables that to some extent are determined by the amount or velocity of thrombin formation. They recently have been (re-)used for the assessment of overall hemostatic function. Some of these methods have the advantage of using full blood. A disadvantage is that the indicating substance (fibrinogen) may increase when TG decreases, eg, in active thrombosis under anticoagulant treatment. It may be extremely confusing that curves are obtained that resemble real TG curves but are not.

Applications of thrombin generation measurement

Thrombin generation measurement has been shown to be a useful tool in several different domains.

Platelet-plasma interactions

Thrombin generation has been instrumental in unveiling the role of platelet receptors in the production of a procoagulant surface by platelets. In short, a role of GPIb/IIia, of GPIb/V, and vWF [28•] and of GPI (collagen), as well as of the PAR receptors, has been demonstrated (see Hemker and Lindhout [43] for a review).

Detection and quantification of thrombotic tendency

Deficiencies of proteins S or C are readily recognized when TM is added to the plasma, factor V Leiden as well [44,45]. APC resistance, either acquired (oral contracep-

Table 1. Normal values and variability

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Average (pop.)</th>
<th>StDev (pop.)</th>
<th>CV (pop.)</th>
<th>CV (ind.)</th>
<th>CV (exp.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPP</td>
<td>36</td>
<td>1879</td>
<td>284</td>
<td>15</td>
<td>4.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>458</td>
<td>60</td>
<td>13</td>
<td>5.5</td>
<td>4.5</td>
<td>5</td>
</tr>
<tr>
<td>Lag (min)</td>
<td>3.1</td>
<td>1.4</td>
<td>47</td>
<td>8.1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>PRP</td>
<td>24</td>
<td>1878</td>
<td>371</td>
<td>22</td>
<td>7.8</td>
<td>3</td>
</tr>
<tr>
<td>ETP (nM.min)</td>
<td>117</td>
<td>42</td>
<td>35</td>
<td>8.3</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Lag (min)</td>
<td>8.1</td>
<td>1.9</td>
<td>23</td>
<td>9.1</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

CV, ; ETP, endogenous thrombin potential; PPP, platelet-poor plasma; PRP, platelet-rich plasma.
Thrombin generation also solves the long-standing enigma of the prothrombotic anticoagulant in lupus erythematosides. It has been shown that this antibody prolongs the lag phase (ie, is anticoagulant in clotting tests) but induces TM and APC resistance [46••].

Detection and quantification of bleeding tendency
In deficiencies of factors II, V, VII, VIII, IX, X, and XI, it has been demonstrated that TG is diminished in PPP and that clinical bleeding is observed at ETP values less than 30% [3••,47].

The thrombasthenias of Glanzmann [48] and Bernard-Soulier [28•], as well as severe thrombopenia, show a moderately diminished TG in PRP. Von Willebrand disease, unless accompanied by severe factor VIII deficiency, shows normal TG in PPP but decreased TG in PRP [20].

Control of procoagulant therapy
As expected, restoration of the factor VIII level of hemophilic plasma restores TG, as does DDAVP treatment in mild hemophilia and vWD [49]. More interesting: inhibitor bypassing therapy with either factor VIIa, or this factor in combination with other factors (Feiba), can be monitored with TG [50•,51••].

Control of antithrombotic therapy
Under oral anticoagulation the incidence of bleeding increases as soon as the international normalized ratio drops below 3 [52], which is equivalent to ETP = 20% [36•]. Heparins, including the low-molecular-weight types, inhibit TG primarily by increasing thrombin breakdown [53•]. Twofold prolongation of the aPTT corresponds to ~80% inhibition of the ETP [54•]. TG is the only available method to combine the quantified effect of heparin and vitamin K antagonists or other anticoagulants.

Platelet “aggregation” inhibitors in general such as abciximab [48], clopidogrel [55], and aspirin [56] also inhibit TG to a certain degree. This is not to say that inhibition of aggregation as such would not have an—or even be the main—antithrombotic action. It is an interesting possibility that through decreasing the size of the platelet aggregate, the volume in which thrombin can form undisturbed by flow is also diminished.

Pharmacologic research
As yet we have not encountered any antithrombotic, either anticoagulant or antiaggregant, that did not inhibit TG [5]. We can assume that any drug that inhibits TG to ~50% of normal will show an antithrombotic effect at an acceptable bleeding risk. By introducing TG as an intermediate step between the biochemical experiments and thrombosis models in experimental animals, we can significantly diminish the latter, especially in dose-finding experiments. The test can also be used to assess the effects of a candidate molecule in volunteers.

Conclusion
To understand thrombin generation, we have to measure thrombin generation, the whole thrombin generation, and nothing but thrombin generation—under conditions as close as possible to those in vivo. This offers a wealth of information that is not otherwise available. Measurement in an undisturbed fibrin clot in which activated platelets are fixed, as is possible with fluorogenic substrates, probably resembles the situation in a hemostatic plug or thrombus more closely than stirred systems do. The calibrated automated technique makes it possible to obtain a graph of thrombin concentration against time in real time in up to 24 parallel experiments. Whole blood measurement is as yet technically impossible.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

1. Of special interest
2. Of outstanding interest


Thrombin generation was measured in PRP from hemophilia A and hemophilia B patients. Platelets increase ETP in the hemophilias. There was an almost linear relationship between increasing platelet count and thrombin generation, reaching a limit at 1011 platelets/L. The influence of platelets diminishes with increasing concentration of either FVIII or FIX.

present-day concepts of TG via the TF pathway and its regulation, seen from a personalized standpoint.


• This review provides a summary of the evolution of knowledge with respect to present-day concepts of TG via the TF pathway and its regulation, seen from a personalized standpoint.


24. Allen GA, Hoffman M, Roberts HR, Monroe DM 3rd: 2004. • The dose-effect relations are determined between the concentration of a number of clotting factors and TG in a system consisting of platelets and TF-bearing monocytes and purified clotting factors. TG increases linearly with prothrombin concentration but reaches an upper limit at minimal concentrations of the other clotting factors. The authors explain this in terms of enzyme kinetics of the clotting process. We consider it to demonstrate diffusion limitation of cell-bound TG.


• Induction of fibrin polymerization during the lag phase of TG by a snake venom enzyme induces an immediate burst of TG that is inhibited by a monoclonal antibody against GP Ib. Inhibition of polymerization decreases TG. So polymerizing fibrin interacts with VWF so as to activate GP Ib and produce platelet procoagulant activity.


• Thrombin substrate binding to fibrinogen is mediated through exosite I. Nonsubstrate binding of thrombin to fibrin occurs at low affinity in the fibrin E domain and at high affinity to a variant gamma chain, found in ~15% of the fibrinogen molecules (fibrinogen 2). Fibrin formation (antithrombin I) thus inhibits the appearance of thrombin in the fluid phase and “high-affinity” thrombin-binding plays a dominant role in this process.


• By using a “slow” fluorogenic thrombin substrate and continuous comparison to a simultaneously run calibrator, TG can be monitored automatically, on line, in clotting PPP or PRP. The resulting thrombogram in PPP measures hypocoagulability (hemophilia, oral anticogulants, heparins, and heparin-like), direct inhibitors), and hypercoagulabilities (AT deficiency, prothrombin hyperexpression, protein C and S deficiency, factor V Leiden, oral contraceptives). In PRP it is diminished in thrombopathies, in von Willebrand disease, by antibodies blocking GPIb-IIIa or GPIIb, or by antiplatelet drugs like aspirin and clopidogrel.


• Whole blood thrombelastography was used according to the traditional technique but the data were mathematically treated to resemble TG curves. Changes of these curves appeared to be dependent on the nature and severity of the hemostatic deficit in hemophiliacs and could be normalized with recombinant factor Vlla. The test also measures thrombolyis.


• Reports a technique for testing ex vivo blood flow. Aggregation and the explosive TG result in occlusion. Occlusion time was dose-dependently inhibited by monoclonal antibody against GPIb, auroin ticarboxylic acid, monoclonal antibody against GPIb-IIIa, a GP Ib/IIIa antagonist, argatroban, and anti-WFJ, but not by antibino.


• Using thrombography, APC resistance can be demonstrated in patients with lupus anticoagulants. A long time lag is observed before the thrombin burst (lupus anticoagulant effect) together with a marked inability of APC to diminish the thrombin activity. The effects were more outspoken in the presence of phospholipids from patients’ platelets than with added phospholipids.


• The relation between clotting factor concentration, the ETP, and the severity of bleeding was investigated in patients with congenital deficiency of factors II, V, VII, X, XI, and XII. In all the patients with severe bleeding, the ETP was less than 20[ref] of normal. Bleeding tendency was absent or mild in patients with an ETP of 30[ref] or higher.


• A TG assay may serve for using the kinetics of inhibitor bypassing agents during treatment and possibly for predicting responses to treatment.
A TG assay enables the pharmacodynamic and pharmacokinetic properties of factor VIII-inhibitor bypassing therapies to be monitored, thus helping to optimize treatment.

For any type of heparin, the capacity to inhibit the coagulation process in plasma is primarily determined by the concentration of the AT-binding pentasaccharide with 12 or more sugar units at its nonreducing end, i.e., the structure that induces antithrombin activity. Antifactor Xa activity hardly influences either ETP or APTT.

In 12 volunteers, 9000 IU of four heparins of different molecular weight distribution was injected. The aPTT showed the effect of heparin in 34 of the samples; the ETP in 80. Relative to the baseline value of the individual, the heparin effect was recognized by the aPTT in 55 of the cases and by the ETP in 98. There were no large differences between the different types of heparin.
