Modified Thrombin Generation Assay: Application To The Analysis Of Immunoglobulin Concentrates

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Modified Thrombin Generation Assay: Application To The Analysis Of Immunoglobulin Concentrates

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Abstract

Intravenous immunoglobulins cause thromboembolic events at a frequency of 2-3%. Complications of this nature can be life threatening. Nevertheless, a test for thrombogenicity such as non activated partial thromboplastin time is not mandatory for these drugs. As a result of recent incidents we have performed experiments to evaluate a modified thrombin generation test for its ability to detect minute amounts of thrombogenic impurities in intravenous immunoglobulin preparations. We were able to show that this modified thrombin generation test is more sensitive than the Ph. Eur. assay for activated coagulation factors.

Introduction

Human plasma derived medicinal products are essential for the treatment of many disorders, hereditary or acquired. Examples are coagulation factor VIII and factor IX (FVIII, FIX) concentrates for the treatment of haemophilia A and B (1), prothrombin complex concentrates (PCC) for the reversal of oral anticoagulation (2), intra venous immunoglobulins (IVIG) for the treatment of immune deficiencies (3), or albumin for the treatment of e.g. severe burns or liver failure (4).

In the past, certain products have been found to be associated with thromboembolic events (TEE) of which FIX concentrates and PCC have been prominent examples (5-8). Therefore, a thrombogenicity test (non activated partial thromboplastin time, NAPTT) must be passed before batch release of these products is granted. In general, this test is performed by adding the substance to be investigated to normal citrated plasma, followed by addition of phospholipids and calcium, and determining the time clotting occurs. An alternative in vitro test, the thrombin generation test (TGT) is increasingly being explored for detecting thrombogenic agents in plasma derived products. Typically, the TGT is performed by mixing citrated plasma with the substance to be investigated, adding phospholipids, minute amounts of tissue factor, and a specific, slow reacting fluorogenic peptide substrate for thrombin (9-11). Thrombin formation is then initiated by the addition of calcium and monitored with a fluorescence photometer. This test has the advantage of giving information on the amount of thrombin formed, and the time it takes to reach peak levels of thrombin. These events continue after the time of clotting, the result obtained by NAPTT. Under suitable conditions, the effect of inhibitors or imbalances in clotting factors on the clotting cascade may also be determined (12, 13).

Thus, it appears the TGT is able to detect modulators and activators of the coagulation cascade.

In plasma used for NAPTT and TGT, preactivated factors (FXIIa and FXIa) of the coagulation cascade are already present in conventional citrated plasma before start of the reaction due to inevitable events during blood collection. In the NAPTT this results in typical clotting times of the citrated plasma of 200-350 seconds. This clotting normally occurs at a time when only ~5% of the prothrombin is converted to thrombin, while the main thrombin burst is yet to come. In the TGT, the moment of clotting is normally invisible, but the time to the corresponding thrombin concentration may be derived from the TGT curve when suitable calibrators are used (14, 15).

The clotting time in NAPTT, and the time to maximum thrombin in a thrombin generation experiment are also influenced by the initiator concentration employed. To obtain maximum sensitivity, tissue factor concentrations in a typical TGT have been reduced to the low to sub picomolar range (16, 17). Corn trypsin inhibitor (CTI) may be added to blood already during collection in order to inactivate FXIIa specifically (18). This is to prevent FXI activation and results in FXIa free or at least FXIa poor plasma, as this factor is known to play an important role in the amplification of the clotting cascade (19).

Recently, we were confronted with the need to detect trace amounts of coagulation activators in IVIG preparations as some of these preparations had apparently caused thromboembolic complications in patients.

It was thought that a system based on the TGT, which we have applied in the past for the analysis of certain coagulation factor concentrates (10, 13) might be suitable. IVIGs normally do not contain high concentrations of coagulation factors due to their manufacturing procedure, but eventually contain traces of FXI and/or its activated form FXIa (20). We
decided therefore to modify our established TGT with the intention to detect even minor quantities of potential activators such as FXIa in these preparations. Since traces of FXIa are expected to be present in conventionally prepared citrated plasma, we considered it prudent to use plasma free of FXIa. In addition, we considered it useful not to add any exogenous activator to this system but make the initiation of coagulation absolutely dependent upon the substance to be investigated. We hereby report the outcome of our study and possible implications on the evaluation of IVIGs (and perhaps other biomedicines).

Materials & Methods

Materials

Blood collection tubes were from Sarstedt (Nuembrecht, Germany). CTI and FXIa were purchased from Calbiochem (Merck Biosciences, Darmstadt, Germany). Cephalin (phospholipid component of PTT reagent) was from Roche Diagnostics (Mannheim, Germany). Phospholipids containing tissue factor (Innovin) was from Siemens Healthcare Diagnostics (Eschborn, Germany). The fluorogenic thrombin specific peptide substrate Boc-Val-Arg-AMC (B-VR-AMC) was obtained from Bachem (Weil am Rhein, Germany). All other chemicals were of the highest grade purity available from different suppliers. Microplates were from Nunc (Wiesbaden, Germany), the fluorescence photometer (Fluoroscan Ascent FL) was from Thermo electron (Dreieich, Germany). The BCS-XP coagulation analyzer was from Siemens Healthcare Diagnostics (Eschborn, Germany). IVIGs and albumin- solutions represent the spectrum of preparations submitted to our institute for batch release control.

Methods

Plasma was obtained from healthy individuals by clear venepuncture and carefully drawing nine volumes blood into one volume 106 mM sodium citrate supplemented with CTI in a final concentration of 100µg/ml. After centrifugation for 20 minutes at 800xg at room temperature, the upper half of the supernatant was withdrawn and centrifuged again at 4000xg for 20 minutes at room temperature. Again, the upper half of the supernatant was collected and frozen in small aliquots at -80°C. TGT was performed in triplicates according to published procedures (10, 15) with modifications as follows: 0.25 µl of a 5% solution of the IVIG preparation to be investigated was added to 25 µl Plasma obtained as described above. Human albumin solutions were included in some experiments as control, because one IVIG- preparation is on the market which contains high concentrations of albumin. The reaction mixture was made up to 50 µl by adding buffer containing Cephalin and the substrate B-VR-AMC. After 5 minutes preincubation at 37°C, the reaction was started by adding 50 µl prewarmed CaCl2- solution in buffer. Thrombin generation was monitored for 1 hour by measuring the developing fluorescence every 20 seconds. A blank curve (containing all ingredients plus EDTA in excess to calcium) was subtracted from all measurements. A dilution series of FXIa (and in some experiments tissue factor) served as calibrator.

NAPTT was essentially performed as described in the general chapter “2.6.22. Activated coagulation factors” of the Pharmacopoeia Europaea (Ph Eur). Briefly, 30 µl human virus inactivated pooled plasma (Octaplas LG, Octapharma, Dessau, Germany) were mixed with an equal volume of Cephalin. After 60 s at 37°C, 30 µl of 1:10 and 1:100 sample dilutions in Tris buffer pH 7.5, and 30 µl of 25 mM CaCl2 were added and the clotting time measured. Assays were performed using the BCS-XP coagulation analyzer (Siemens Healthcare Diagnostics).

Results

Thrombin generation in plasma prepared with and without CTI

As plasma collected in presence of CTI is not commercially available, we compared the influence of CTI on thrombin generation in an experiment in plasma obtained from a healthy donor. The plasma was prepared in presence and absence of CTI (100µg/ml), respectively. The reaction was triggered by addition of tissue factor (0- 5 pM final concentration). The result is presented in illustration 1. With increasing tissue factor concentrations the time to peak shortens in plasma prepared without CTI. However, the amount of thrombin generated is not significantly influenced in these plasmas. As has been reported already by other groups, at high tissue factor concentrations there is only a limited difference in TGT between plasmas prepared with and without CTI, whereas at lower tissue factor concentrations this difference becomes more pronounced (22, 23). This effect is most likely explained by the inhibition of activated FXII by CTI, and thus avoidance of FXI activation during blood collection.

Thrombin generation in plasma with FXIa as the trigger

The result above suggests that plasma prepared in absence of CTI contains enough FXIa to trigger the coagulation cascade. This prompted us to examine the
effect of FXIa as initiator of thrombin generation in plasma prepared in presence of CTI without tissue factor addition. The result of this experiment is shown in illustration 2. It appears that triggering the reaction by FXIa results in generation of a constant amount of thrombin when a certain threshold level of FXIa (~1 pM) is exceeded. On the other hand, the time to peak (TTP) varies with FXIa concentration over the entire range tested. This range was determined in preliminary range finding experiments with selected IVIG- concentrates (including the product which caused TEE as well as products from different suppliers which did not cause complications). Illustration 3 shows a good correlation between FXIa-concentration and TTP.

**Influence of IVIGs and albumins on thrombin generation**

Using CTI treated plasma we have attempted to answer the question: Do IVIGs or some albumin solutions available on the German market contain factor(s) able to trigger the coagulation cascade? Solutions to be investigated were adjusted to a concentration of 5% (based on the labelling). These samples were then added to CTI- plasma and the thrombin generation experiment started. As shown with purified FXIa as the trigger (illustration 2), we also observed differences in the time to peak in the experiments with IVIG concentrates and a decrease of peak height only in the IVIG and albumin preparations showing very long lag times (~20 minutes). A comparison of IVIGs from different manufacturers is presented in illustration 4. All IVIGs from the manufacturer who produced the preparation which caused TEE (manufacturer A) show a similar behaviour of pronounced peaks. However, the batches in this group differ significantly with respect to the lag time. In contrast, all products tested from the other manufacturers show strikingly longer time to peak and a remarkably reduced peak height.

**Influence of IVIGs and albumin on NAPTT**

To compare the results obtained by the modified TGT with the established NAPTT, we tested the IVIGs investigated above and albumins by NAPTT. These results are summarized in illustration 5 together with results obtained by TGT (expressed as TTP). Three groups can be identified from this analysis: One group (albumins and IVIGs from competitors) is clustered around 250 seconds as determined by NAPTT or 40 minutes by TTP. A second group (all from manufacturer A) shows clotting times between 220 and 270 seconds and 15 to 25 minutes in TTP. The third group (also from this manufacturer) fails the test for activated clotting factors NAPTT (less than 150 seconds). This clotting time corresponds to 10 minutes or less in TTP.

**Discussion**

In a conventional clotting experiment such as partial thromboplastin time (PTT) or NAPTT, one measures the time a sample takes to clot. This is only part of the relevant information as several events triggered by initiation of the clotting cascade are still to follow. During the last decade, thrombin generation measurements have become more and more attractive, as it could be shown that in the course of a TGT, information is gathered of events even after clotting has occurred. Some examples are reviewed by Hemker et al. (9). Despite many merits TGT has earned, this test is not employed as a routine clinical test, probably because of poor standardization (24, 25).

Since we wanted to preclude any preactivation, we avoid using vacuum containers for blood collection and prefer cautious manual aspiration (15). The risk of activation of clotting factors during blood harvesting is further reduced by including CTI as inhibitor of FXIa. Principally, the use of FXI depleted plasma could provide a means to prevent FXIa formation. However, as long as the initiator of coagulation in the sample to be tested is unknown, we consider it more appropriate not to exclude molecules from the analysis which may be needed for downstream events. FXI is considered to play an essential role during amplification mechanisms in the clotting cascade which we do not believe should be left out when assessing the whole process of coagulation. These thoughts led us to a modification of the TGT based on plasma prepared in presence of CTI and the TGT performed without tissue factor addition. For the special purpose of detecting thrombogenic components in IVIGs or albumins, we omitted tissue factor as initiating reagent, since this molecule could mask triggering effects caused by components in the sample under investigation. This assumption seems justified when CTI inhibited plasma is compared to uninhibited plasma (illustration 1). CTI uninhibited plasma may contain enough trigger molecules (presumably FXIa) to initiate the coagulation cascade.

Although the agent in IVIGs responsible for triggering the coagulation cascade in affected patients and in our in vitro model is not known as at now, we have chosen FXIa as reference molecule for two reasons: i) FXI and presumably FXIa are known contaminants in IVIG-preparations (20). ii) FXIa shares a remarkable similarity with the unknown component in IVIGs in our modified TGT: TTP of 20 minutes or less are not
accompanied by increasing amounts of thrombin with either FXIa or IVIGs as initiator. This corresponds to ~1 pM FXIa and discriminates it from tissue factor (illustrations 1, 2, 3). As long as the exact nature of this molecule is not clarified we prefer to use the term “FXIa equivalents”.

What part do IVIGs play in our modified TGT? The answer to this question is given in illustration 4, where three representative batches produced by manufacturer A are compared to four batches from other manufacturers. The three batches from manufacturer A are selected from a total of 19 batches tested so far: the first batch represents the highest initiator concentration, followed by one with intermediate concentration, and the batch with the lowest concentration of initiator.

The NAPTT is mandatory for e.g. FIX- concentrates and PCC but not for IVIGs. This test was performed according to Ph. Eur. procedures with conventionally prepared citrated plasma without CTI (see methods). The comparison of the NAPTT and the TGT is shown in illustration 5. A clear contrast between samples from manufacturer A and samples produced by other manufacturers (IVIGs and albumins) is obvious only in TGT: One group (from manufacturer A) shows clotting times of 150 seconds or less compared to TTP of around 10 minutes. Another group (also from manufacturer A as well as from others) shows NAPTT clotting times of between 220 seconds and 270 seconds (the reference plasma clotted after 275 seconds). These samples are poorly differentiated by NAPTT but very well separated by TGT into two groups: one group with TTP of ~20 minutes (from manufacturer A) and the other with TTP of ~ 40 minutes (other manufacturers and blank plasma as the reference). It is evident that all products from manufacturer A contain measurable amounts (by TGT) of thrombogenic agents although at different concentrations.

Our results show that both tests, NAPTT and the modified TGT, are able to detect thrombogenic substances when a certain concentration is exceeded. This concentration is defined in the NAPTT as clotting times less than 150 seconds for PCC (corresponding to ~50 pM FXIa equivalents). Although TGT is not an official test for thrombogenicity, it is able to detect even lesser amounts of thrombogenic agents. Its kinetic evaluation allows a finely graded estimation of thrombogenic agents down to below 1 pM FXIa equivalents (illustration 3).

It would be of interest to study the effect of using CTI inhibited plasma in the NAPTT and find out if this test can be improved with respect to sensitivity analogous to TGT. This assumption appears justified as NAPTT does not involve trigger molecules for coagulation other than those contained in the sample under investigation. In this respect it is directly comparable to the modified TGT. We could well envisage that such a modification of NAPTT or a derivative of our modified TGT may become a substitute to the approved NAPTT in the future and may be applied to the analysis of other medicines such as IVIGs as demonstrated in this communication.

Abbreviation(s)
TEE: thromboembolic events  
NAPTT: non activated partial thromboplastin time  
TGT: thrombin generation test  
FXI(a): (activated) factor XI  
CTI: corn trypsin inhibitor  
IVIG: intravenous immunoglobulins  
TTP: time to peak  
TTC: time to clot

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Authors Contribution(s)
Claudia Grundmann, Manuela Kusch, Stefanie Keitel performed the experiments. Andreas Hunfeld, Susanne Breitner Ruddock, Rainer Seitz, Herbert König designed the research, analyzed and interpreted the results, and wrote the paper

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Illustrations

Illustration 1

Influence of tissue factor and CTI on thrombin generation

Plasma prepared in presence of CTI (symbols) and plasma prepared in the absence of CTI (lines) was triggered by increasing amounts of tissue factor (TF) and TGT was performed as described in methods.

Continuous line, squares: 5 pM TF, disjointed line, diamonds: 1.25 pM TF, dotted line, triangles: 0.3125 pM TF, disjointed line with dots, circles: 0 pM TF.
Illustration 2

Influence of varying concentrations of FXIa on TGT

TGT was performed as described in methods with CTI treated plasma. FXIa (1000-0 pM) served as trigger. For clarity, only every second concentration is illustrated here (TTPs of the complete experiment are shown in illustration 3).

- 500 pM FXIa: continuous line with triangles
- 125 pM FXIa: continuous line with squares
- 31.25 pM FXIa: continuous line with diamonds
- 7.8 pM FXIa: continuous line with crosses
- 1.95 pM FXIa: continuous line
- 0.49 pM FXIa: disjointed line
- 0.12 pM FXIa: dotted line
- 0 pM FXIa: discontinuous line with dots
TGT was triggered with FXIa (1000-0 pM) as in illustration 2. All tested FXIa concentrations are plotted vs. TTP in a double logarithmic plot.
Illustration 4

IVIGs as initiators of IIa generation

Selected IVIGs from manufacturer A and IVIGs from other manufacturers are compared with respect to their influence on TGT. Continuous line with squares: IVIG from manufacturer A ("high activity"); continuous line with diamonds: IVIG from the manufacturer A ("medium activity"); continuous line with triangles: IVIG from manufacturer A ("low activity"); lines without symbols: IVIGs from competitors.
Illustration 5

Comparison of NAPTT and TGT

NAPTT was done according to Eur. Pharm. TGT was performed as described in methods. The times to clotting (TTC) obtained by NAPTT with the respective concentrates were plotted against their corresponding times to peak (TTP) obtained by TGT.

Triangles: IVIGs from manufacturer A.
Squares: IVIGs from several other manufacturers.
Diamonds: albumin concentrates.
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