Identification of prothrombin as a major thrombogenic agent in prothrombin complex concentrates

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Prothrombin complex concentrates (PCC) were compared in an *in vitro* test system for thrombogenicity (thrombin generation assay) employing plasma from coumarin-treated patients. Among these concentrates one had a proven history of thrombogenicity, whereas the remainder did not cause such fatal casualties in the past. Investigations into the thrombogenic component were performed by spiking experiments in which we biased a typical PCC without reported thromboembolic complications into one with a performance in the thrombin generation assay like that with a proven history of thrombogenicity. Hereby, it was possible to identify prothrombin as the most plausible thrombogenic component. Additional experiments performed with anticoagulant components (antithrombin together with heparin) resulted in a perfect reversal of the observed *in vitro* thrombogenicity. Our *in vitro* observations corroborate on an experimental basis the widespread medicinal usage of antithrombin administration as a regimen for the avoidance of thromboembolic complications during treatment with PCC and related products, and *vice versa*. Our observation casts doubts upon the widely accepted idea of activated factor IX as the thromboembolic agent in PCC. Also, our finding may be taken as an example for the feasibility of this test system as an *in vitro* model for thrombogenicity. *Blood Coagul Fibrinolysis* 15:405–411

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Introduction

Prothrombin complex concentrates (PCC) were originally manufactured for the treatment of factor IX (FIX) deficiency, called haemophilia B [1]. They have also successfully been used for the treatment of patients with acquired coagulation disturbances like liver diseases causing a deficiency in vitamin K-dependent clotting factors [2], and for the treatment of heavy blood loss caused by polytrauma or during surgery [3]. It was further shown that PCC are superior to fresh frozen plasma for fast reversal of oral anticoagulation by coumarins [4,5]. Since the availability of high-purity factor IX concentrates (HPFIX), the use of PCC for the treatment of haemophilia B has been abandoned and almost restricted to the emergency reversal of oral anticoagulation. Even with these benefits certain PCC showed severe disadvantages during their time of use. One major drawback was the fact that certain PCC bear the risk of thromboembolic complications such as thrombosis, myocardial infarction, or disseminated intravascular coagulation in patients with acquired coagulation disturbances [3,6–9] and in haemophilia patients with inhibitors [10,11]. This inherent risk of thrombogenic complication has been attributed to activated clotting factors [12,13], contaminating phospholipids [14], or to the phenomenon of zymogen overload [15]. The latter explanation refers to the fact that for the treatment of haemophilia B only FIX needs substitution, whereas the other components of PCC result in supraphysiological vitamin K-dependent plasma protein levels. With the availability and the increased use of HPFIX during treatment of haemophilia B a reduced risk of thromboembolic complications was observed in humans [16–19] and in animal experiments [20,21]. This has led to the proposal that activated factor IX (FIXa) is the major thrombogenic trigger in PCC as compared with HPFIX [22]. The evidence for this proposal was based not on direct evidence obtained from PCC, but on the analysis of activation markers in blood obtained from patients [22] or in animal experiments [23]. Recently, we have used a different and more direct approach to clarify the role that FIXa plays in thrombogenicity, by which it was possible to exclude FIXa as the major trigger for thrombogenicity [24].

At that time, however, we were unable to positively identify the major cause for thromboembolic complications brought about by PCC. Consequently, we have searched for an approach to solve this important, though open question. In order to mimic the current

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therapeutic use of PCC in an *in vitro* system, we investigated the influence of various PCC on therapeutically anticoagulated plasma (i.e. plasma obtained from patients treated with coumarins). This analysis was undertaken in an *in vitro* model for thrombogenicity [25–27]. We now report the results of these investigations by which we compared the performance of PCC, one with 'proven history' of thrombogenicity and several without, in this *in vitro* model for thrombogenicity. We also demonstrate how we succeeded to modify a typical member of the latter group such that it exhibits a performance like the former by adding defined amounts of isolated coagulation proteins to the PCC. Using this approach it was possible to investigate the respective roles of several PCC components in thrombin generation and, hence, in its potency to cause overshooting of coagulation to occur.

Materials and methods

Materials

PCC were obtained from the respective manufacturers for the purpose of batch release control. The concentrates under investigation were Beriplex (Aventis Behring, Marburg, Germany), Oktaplex (Oktapharma, Vienna, Austria), several prothrombinkomplex human (German Red Cross, Springe, Hagen, Germany), and Prothromplex (Baxter/Immuno, Heidelberg, Germany). Due to the official duties of our institute as the German control authority for blood products, the results obtained with the respective products, proprietary names, and manufacturers are kept anonymous. The different PCC and their respective coagulation factor contents as declared by the manufacturers are presented in Table 1. Normal plasma was from Baxter/Immuno (Vienna, Austria) or prepared from blood donations from healthy volunteers of our institute. Coumarin anticoagulated plasmas (CAP) (International Normalized Ratio, 2.5–3) were obtained from members of our institute that were treated with Marcumar (Roche-Pharma, Reinach, Switzerland) Phenprocoumon or comparable drugs due to different causes. Additional CAP were a kind gift from Dr Kirchmaier, (German Clinic for Diagnostics, Wiesbaden, Germany) and Dr Schinzel (2nd Medical Clinic, University of Mainz, Germany). Human serum albumin was obtained from Baxter/Immuno (Austria). Antithrombin (AT) (Atenativ or Kybernin) was obtained from Pharmacia (Stockholm, Sweden) and Aventis Behring, respectively. Coagulation factor II, factor VII (FVII), FIX, factor X, and their respective activated companions were purified from human plasma as zymogens [28,29], and were activated where needed according to published procedures with minor modifications [24]. Recombinant soluble human tissue factor was cloned and over-expressed in and purified from recombinant *Escherichia coli* by a simple two-step procedure involving Ni^{2+}-nitrilo tri-acetic acid chromatography and cation exchange chromatography on a Poros SP column. Activated factor VII was derived from FVII by circulating it over a column prepared from recombinant soluble human tissue factor immobilized to CNBr-Sepharose in the presence of activated factor X, phospholipids, and calcium. The thromboplastin reagent (Innovin) was obtained from Dade-Behring (Marburg, Germany). Boc-Val-Arg-AMC (a poor but rather specific thrombin substrate [30]) was obtained from Bachem (Heidelberg, Germany). Phospholipids (Cephalin) and heparin (Liquemin) were from Boehringer/Roche (Mannheim, Germany).

Chromatographic equipment (FPLC system) and chromatographic media (Sephadex G 25, Q-Sepharose FF, Mono Q, Mono S, and CNBr-Sepharose) were from Amersham/Pharmacia (Heidelberg, Germany). Heparin–agarose and low molecular weight heparin were from Sigma (Deisenhofen, Germany), blue agarose was from BioRad (Munich, Germany), Ni^{2+}-nitrilo tri-acetic acid–agarose was from Qiagen (Hilden, Germany), Poros SP media and self-pack columns were from PE Biosystems (Weiterstadt, Germany). The fluorescence photometer (Fluoroscan Ascent FL) was from Labsystems (Helsinki, Finland) with filters set to 355 nm excitation and 440 nm emission.

Table 1 **Content of active ingredients in prothrombin complex concentrates**

	Factor IX	Factor II	Factor VII	Factor X	Antithrombin	Heparin
PCC a	1	1.3	0.7	1.5	0.015	0.05
PCC b	1	3.6	0.6	1.6	–	0.5
PCC c	1	0.9	0.5	0.9	–	0.35
PCC d	1	1	0.7	1	0.03	0.3
PCC e	1	1	0.3	0.75	–	0.15
PCC f	1	1.3	0.5	1.2	0.05	0.25

Dependent upon the manufacturing process, different prothrombin complex concentrates (PCC) contain different amounts of active ingredients and are available in different package sizes. As they are labelled according to factor IX (FIX) content a comparison is most easily achieved by setting this protein to one. The other components can then be expressed according to their content relative to FIX.
PCC a is explained in detail here for clarity: this PCC contains 500 IU FIX (= 1 × 500), 650 IU factor II (= 1.3 × 500), 350 IU factor VII (= 0.7 × 500), 750 IU factor X (= 1.5 × 500), 7.5 IU antithrombin (= 0.015 × 500), 25 IU heparin (= 0.05 × 500).

Flat-bottom microtitre plates with relatively low background fluorescence (catalog number 269620) were from Nunc (Wiesbaden, Germany).

Methods

Thrombin generation was measured in a total volume of 100 µl in microtitre plates. Fifty microlitres of plasma (normal or coumarin anticoagulated) were pipetted into wells, supplemented with additives under investigation (PCC, PCC with purified coagulation factors, or buffer) and made up to 60 µl with HBS (10 mmol/l Hepes, 150 mmol/l NaCl, 1% human serum albumin, pH 7.4). Then, twofold dilutions in HBS were performed, leaving 30 µl respective plasma/additive mixture in the wells. To each well were then added 20 µl fivefold concentrated working reagent (10 µl phospholipids, 25 pmol/l tissue factor, 2.5 mmol/l Boc-Val-Arg-AMC, in HBS). The microtitre plate was then placed in the reader (preheated to 37°C) and (after 2 min warming phase) the reaction started by adding 50 µl of 20 mmol/l $CaCl_2$ in HBS with the aid of the built-in injector. The wells thus contained in addition to the component under investigation: 25 mIU plasma proteins, 10 µl phospholipids, 5 pmol/l tissue factor, 0.5 mmol/l Boc-Val-Arg-AMC, 10 mmol/l $CaCl_2$ in HBS. The fluorescence signal was typically recorded for 1 h at 20 s intervals. The replicate measurements (from duplicate to fivefold) were checked for (a) congruence and (b) dilution dependence, and corresponding measurements were then averaged in order to smoothen the curves. The data were then imported into a Microsoft Excel data sheet and the first derivative was calculated (ΔF/time). Heat-inactivated plasma (1 h, 56°C) spiked with purified and active site-titrated thrombin served as the calibrator. Note that the plasmas were neither depleted with respect to fibrinogen (by, for example, ancrod) nor were coagulation inhibitors (e.g. Gly-Pro-Arg-Pro) added. This allowed one to determine the coagulation time by carefully inspecting the resulting curves, as coagulation causes a short disturbance of the fluorescence signal. This conclusion was drawn from experiments in which the effect of adding Gly-Pro-Arg-Pro was compared with plasma alone or defibrinated plasma (not shown).

Results

First we conducted initial experiments to determine the amount of one PCC with no known fatal casualty (PCC−) necessary to convert CAP into normal plasma with respect to performance in the thrombin generation assay. We are well aware that clinically it is not necessary to restore full coagulation activity as 30–50% activity are normally considered sufficient. However, normal plasma is the legitimate reference in coagulation studies and the results are more evident under these conditions and easier to compare.

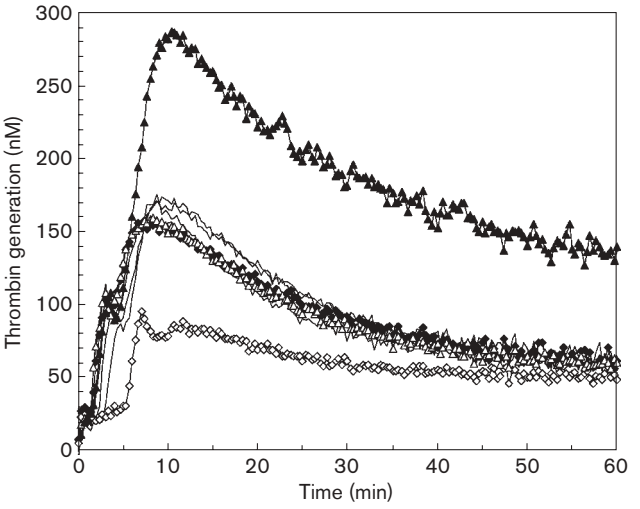
Influence of various PCC on thrombin generation in CAP

The following experiment was then designed to investigate the influence of various PCC from the German market [among these one with known fatal casualties (PCC+)] on CAP and thus to compare PCC− and PCC+ with respect to their influence upon thrombin generation in CAP (Fig. 1). The individual curves of PCC− are hard to discriminate, however; when added in equal amounts they all restore thrombin generation to normal levels, whereas PCC+ leads to a significant overshooting in this respect. Also, it is obvious from Figure 1 that 12.5 mIU PCC− are able to restore full thrombin generation capacity to 25 µl CAP. As 25 µl normal plasma contain 25 mIU vitamin K-dependent zymogens, this indicates that only about 50% of these proteins are functional in CAP.

Which PCC component contributes most to thrombin overshooting

In the following experimental series we addressed the question of which component(s) in PCC+ was (were) responsible for the dramatic difference between this and the other PCC. To elucidate this point we added single purified prothrombin complex components to a typical PCC− in order to mimic PCC+ with respect to its performance in the thrombin generation assay together with CAP. As FIX by convention is the

Fig. 1

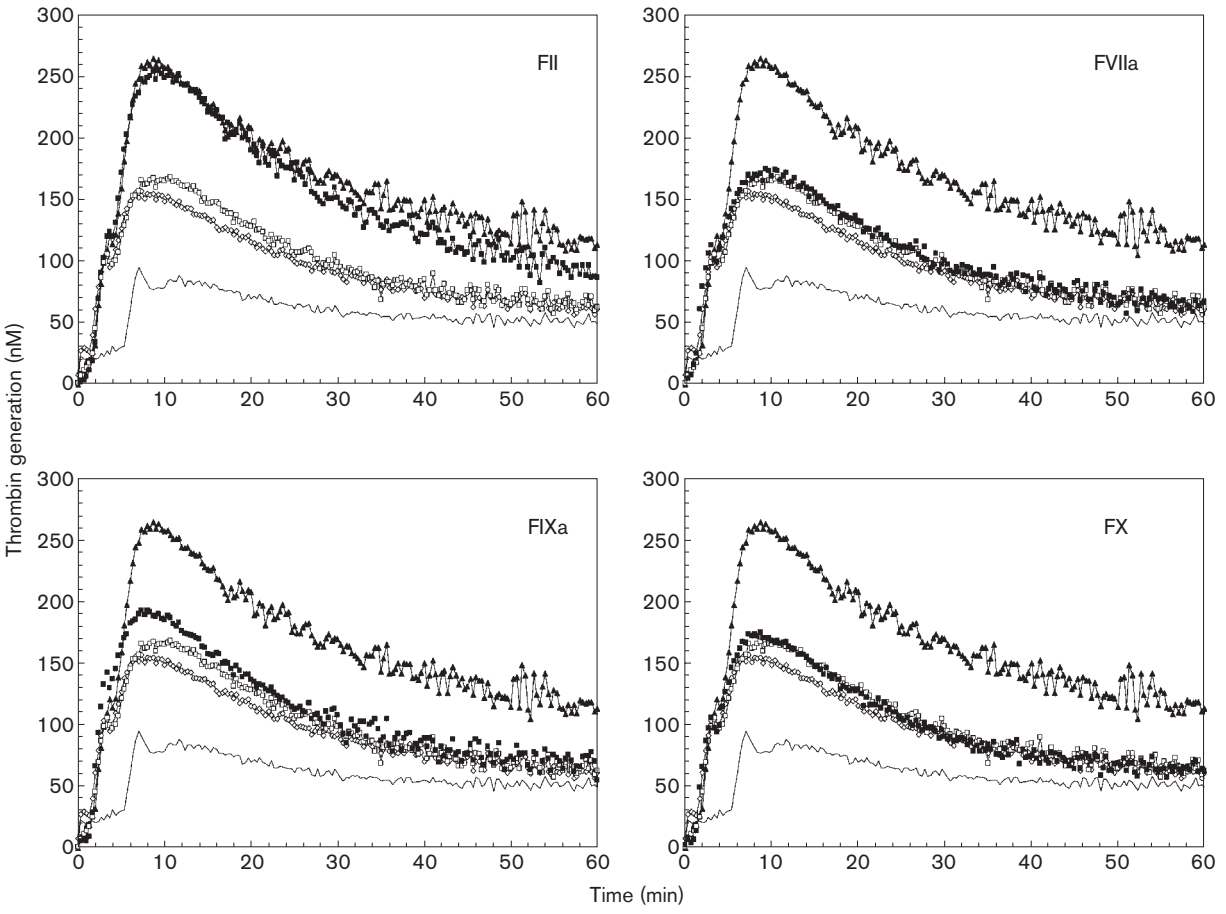


Influence of different prothrombin complex concentrates (PCC) upon thrombin generation in coumarin anticoagulated plasmas (CAP). Thrombin generation was determined with 25 mU plasma as described under Methods in the presence of CAP alone (open diamonds), normal plasma (closed diamonds), CAP plus 12.5 mU PCC a (open triangles), CAP plus 12.5 mU PCC b (closed triangles), CAP plus 12.5 mU PCC c (no symbols), CAP plus 12.5 mU PCC d (no symbols), CAP plus 12.5 mU PCC e (no symbols), and CAP plus 12.5 mU PCC f (no symbols). Only PCC that are utilized in the following experiments are identified here for clarity.

reference protein for PCC, most PCC differ only significantly with respect to the concentrations of the other protein components (see Table 1) and with respect to the amount of activated factors. Based on our detailed knowledge of the components and their respective concentrations in the various PCC, we were able to vary the concentration of the purified proteins added to PCC− in the range given by the respective concentrations as observed in PCC+. The results of this experimental series is illustrated in Figure 2. Although these experiments were conducted in parallel, the results are presented here in separate figures to provide more clarity. Only results obtained with normal plasma, CAP, CAP reconstituted to normal plasma with PCC−, and CAP reconstituted with PCC+, respectively, are shown in each figure to improve the comparison. Zymogen FVII was omitted in this experimental series as it is hardly available in its pure form and, moreover, PCC+ contains even less FVII than PCC−. In addition, the major part of FVII contained in PCC+ is activated (results obtained in our routine tests during batch release). The addition of FVIIa to PCC− up to the rather high levels observed in PCC+ did not result in enhanced thrombin generation when used for CAP reconstitution (Fig. 2, FVIIa). However, we observed a slightly shortened lag time after addition of FVIIa to PCC−. Similarly, neither FIXa (Fig. 2, FIXa) nor factor X (Fig. 2, FX) in concentrations as observed in PCC+ gave a significantly enhanced signal when added together with PCC− to CAP. A completely different result was obtained, however, when prothrombin in concentrations as observed in PCC+ was used together with PCC− for CAP reconstitution (Fig. 2, FII). Thrombin generation is significantly increased, an effect that was proven to be dose dependent (see later).

Besides the ingredients tested in the earlier described experiments, PCC contain additional vitamin K-depen-

Fig. 2



Influence of the main prothrombin complex concentrates (PCC) components upon thrombin generation. PCC with no known fatal casualty (PCC−) (identical to PCC a from Fig. 1) was adjusted to contain factor II levels as observed in PCC with known fatal casualty (PCC+) (FII), activated factor VII levels as observed in PCC+ (FVIIa), activated factor IX levels as observed in PCC+ (FIXa), and factor X levels as observed in PCC+ (FX). Coumarin anticoagulated plasmas (CAP) (no symbols), normal plasma (open diamonds), CAP reconstituted with PCC− (open squares), CAP reconstituted with PCC+ (closed triangles), and CAP reconstituted with PCC− with the respective coagulation factor added (closed squares).

dent proteins (protein C, protein S) that were not investigated in the thrombin generation assay because their concentrations did not differ significantly in the PCC under investigation (unpublished enzyme-linked immunosorbent assay results). Similarly, thrombin and activated factor X were not investigated here as in accordance with Eupharm, both proteases were hardly detectable in all PCC (unpublished results). Phospholipids have also been considered as being responsible for thrombogenicity in PCC [14]. If phospholipids were a contaminant with outstanding thrombogenic potential in PCC one would expect thrombin generation to occur in the thrombin generation assay without exogenously added phospholipids. This, however, was never observed regardless of which PCC was investigated (not shown).

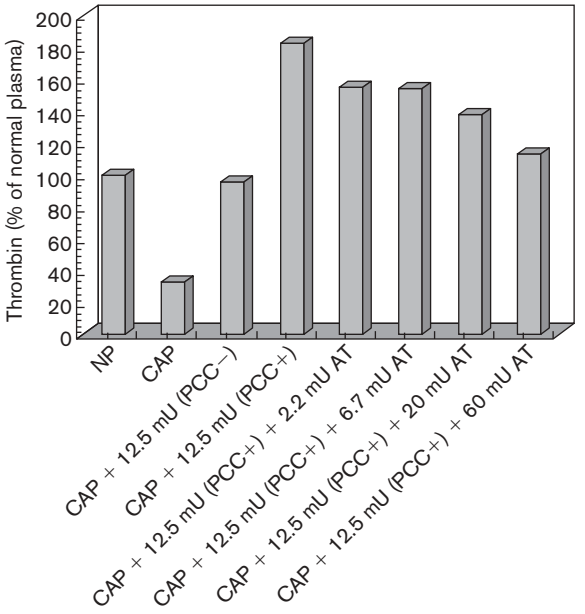
The influence of AT upon thrombin generation in CAP

The fact that some manufacturers add small amounts of AT to their PCC and some do not suggest additional experiments to clarify the role that AT addition to PCC plays in this context. This more so as PCC+ belongs to the group of PCC without AT added while other tested PCC− do contain AT. In this context it is important to realize that all PCC examined in this investigation contain heparin in order to reduce the thrombogenic potential and, moreover, PCC+ containing the highest amount. Thus, a lack in heparin could be excluded as the cause for the thrombogenicity observed with these PCC. In these experiments we added increasing amounts of AT to PCC+, which was used to reconstitute CAP. The result of this experiment is illustrated in Figure 3. It becomes clear from this experiment that the addition of AT to PCC+ leads to a reduction in thrombin generation to levels observed in the absence of excessive prothrombin, as for example in normal plasma or CAP complemented with PCC−. This effect is observed, however, only at AT concentrations that by far exceed those observed in PCC with AT added by the manufacturer (0.01–0.05 IU AT/IU FIX; see Table 1). To reduce thrombin generation to normal levels AT is necessary in amounts that restore the relative physiological concentrations, namely 1 IU AT/1 IU prothrombin. Thus, to 'neutralize' PCC+ by AT addition requires approximately 4 IU AT/ 1 IU PCC as in this particular PCC the FIX/prothrombin ratio is approximately 1/4.

An alternative reference protein for PCC upon thrombin generation in CAP

From the already presented experiments we concluded that prothrombin is the component in PCC with the highest (if not exclusive) impact upon thrombin generation. We therefore reasoned that in order to obtain thrombin generation comparable with that observed with normal plasma, one should vary the PCC+ concentration such that equal prothrombin equivalents were employed as compared with PCC−. Based upon our detailed knowledge of the various PCC and their respective active ingredients we performed experiments with PCC concentrations with the prothrombin content as the reference on this occasion. The result of such an experiment is illustrated in Figure 4. Obviously, a smaller amount of PCC+ is sufficient to restore thrombin generation in CAP to normal levels. More specifically, the same argument is valid as described earlier in the AT experiment (i.e. PCC+ contain approximately 4 U prothrombin/U FIX).

Fig. 3

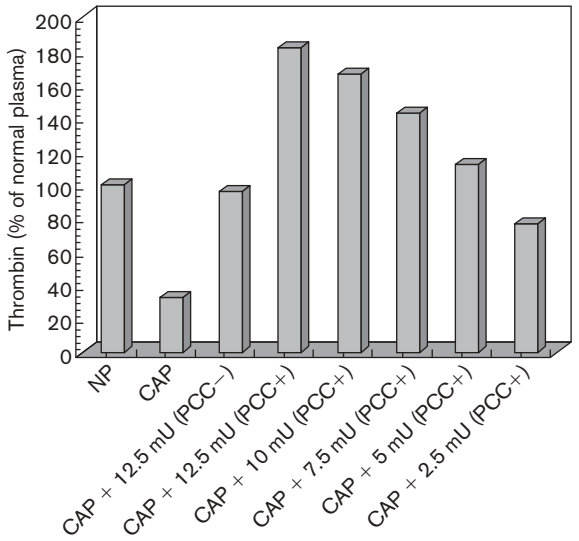


Influence of antithrombin (AT) upon thrombin generation. Thrombin generation was performed as described in Methods and the thrombin generated was calculated. Coumarin anticoagulated plasmas (CAP) is compared with normal plasma (NP), with CAP reconstituted with PCC with no known fatal casualty (PCC−), with PCC with known fatal casualty (PCC+), and with PCC+ in the presence of different concentrations of AT. The thrombin generation obtained under the various conditions is presented as a percentage of that obtained with normal plasma.

Discussion

After we were confident that FIXa is not the thrombogenic agent in PCC [24] we decided to use an *in vitro* model of thrombogenicity in order to positively identify the responsible agent(s). Such a model would enable us to carry out numerous experiments, in which several components of PCC could be evaluated with respect to their influence upon thrombogenicity in normal plasmas and in therapeutically anticoagulated plasmas, such as plasmas from coumarin-treated patients. In addition, it was very helpful that a PCC with a history of fatal casualties was available to us as well as PCC without such a history. These PCC could be compared in such

Fig. 4



Determining the amount of PCC with known fatal casualty (PCC+) sufficient to restore normal plasma thrombin generation. Analogous to Figure 3, thrombin generation was allowed in normal plasma, coumarin anticoagulated plasmas (CAP), and CAP reconstituted with different concentrations of PCC+. The resulting thrombin generation is presented as a percentage of that obtained with normal plasma (NP).

an *in vitro* system with respect to their impact on plasmas from anticoagulated patients.

For this aim it was helpful that not only a survey of the active ingredients was available to us, but moreover we had performed analyses of the respective concentrates during batch release procedures. Thus, we had knowledge not only of which component(s) were present in one PCC but not in the other, but also of how these components differed with respect to their concentration in the respective PCC.

As the *in vitro* model for thrombogenicity we have chosen the thrombin generation assay because it is well established for the analysis of substances with impact on thrombin generation and as a consequence upon the possible fibrin generation. In this regard the assay has gathered some merits during the past 10–15 years [31–33]. In order to make handling easier as well as to increase sensitivity, the assay has been modified in that it is now performed without subsampling and by employment of a fluorogenic peptide substrate for thrombin originally described and catalytically characterized [30]. In addition, we noticed during our initial experiments employing different plasma concentrations that the assay could be scaled down significantly without detrimental effect to its principal significance.

Our attempts to mimic the events that *in vivo* might have led to thromboembolic complications by an *in vitro* system yielded a convincing agreement between thrombin generation curves and the different PCC: only PCC+ gave a signal that deviated significantly from normal plasma when used for CAP reconstitution. This result was confirmed and further extended by experiments that were designed to identify the PCC component responsible for this phenomenon. Activated factors that have been suspected to cause thromboembolic complications [12,13] could be excluded or considered unlikely by recent observations with FIXa [24], by the finding that activated factor II and activated factor X are hardly detectable in these concentrates, and by the successful usage of large amounts of FVIIa to treat haemophilia A in patients with inhibitory antibodies to factor VIII.

Phospholipids have been shown to be a determinant of thrombogenicity in PCC in a rabbit model [14]. However, since then procedures have been introduced to reduce the risk of viral infection by solvent detergent treatment of concentrates. This processing step also reduces phospholipid content in concentrates significantly. In agreement with this theoretical consideration we did not observe thrombin generation without adding phospholipids to the mixture (not shown).

Our results, however, are in good agreement with earlier observations that favour zymogen overload as the main causative agent for thrombogenic potential in PCC [15]. This is even more substantiated by the observation that the physiological ratio between prothrombin and ATIII must be re-established to reduce the thrombin generation to normal levels (Figs 3 and 4; PCC+ contains approximately 4 IU factor II/IU FIX). Also, it was reported very recently that thrombin generation in the presence of FEIBA, an activated PCC, is mainly dependent upon prothrombin [34]. A similar result was reported in 1990 [25] when thrombin generation in newborn plasma was investigated. Taken together, evidence is accumulated that makes the long-term-preferred FIXa as the thrombogenic trigger (reviewed in [35]) in PCC highly unlikely.

Taking into account the results of this investigation, there are two options to reduce the thrombogenic risk of PCC in the future. One option, which is a common clinical practice, is to give AT together with or in advance of PCC [36]. This approach appears to be in accordance with our data as AT, as long as it is in stoichiometric excess over prothrombin, is able to control thrombin generation. In this context we would like to mention the pioneering work of the group of Dr K. Mann, which stated that for 'normal thrombin generation', one might change several coagulation factor concentrations, but should not alter the relative concentrations of AT or prothrombin [37]. However, such a treatment would increase the cost significantly.

As we have shown that PCC+ can be used to restore normal thrombin generation in CAP when its amount is reduced such that the prothrombin content resembles that in PCC−, the (in our view) preferable concept would require that PCC are labelled according to their prothrombin instead of FIX content. Such a proceeding should be preceded by *in vivo* investigations. Such experiments are currently underway in our institute.

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