The Coagulant-Active Phospholipid Content is a Major Determinant of In Vivo Thrombogenicity of Prothrombin Complex (Factor IX) Concentrates in Rabbits

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In vitro evaluation of prothrombin complex concentrates in a thrombin generation assay, using DAPA and purified components of the prothrombinase complex, demonstrated significant levels of coagulant-active ‘phospholipid replacing’ activity. Quantification of this activity showed a significant correlation ($r = 0.8747, p < 0.01$) with thrombogenicity measured in vivo in a stasis model in rabbits. Extracted lipid material retained full phospholipid replacing activity in the in vitro assay. Thin-layer chromatographic characterization confirmed the presence of phospholipids with known coagulant activity in vitro. In vivo, the extracted material was nonthrombogenic but augmented the thrombogenicity of purified factor Xa. Substitution of a synthetic coagulant-active phospholipid (phosphatidylcholine-phosphatidylycerine lipid vesicles) for the extracted phospholipid produced a similar augmentation of factor-Xa-induced thrombogenicity in vivo. It is concluded that the coagulant-active phospholipid content of prothrombin complex concentrates is a major determinant of thrombogenicity but requires the presence of activated clotting factors for its expression in vivo.

We have previously reported a lack of correlation between the activated clotting factor content of some prothrombin complex concentrates and their subsequent thrombogenicity measured in vivo in a stasis model in rabbits, thus challenging the assumption that the former was necessarily an adequate determinant of the thrombogenic potential of all available products. Certain plasma pools were subsequently demonstrated to contain a thrombogenic component that was not itself an activated clotting factor, but expressed its latent thrombogenicity in vivo when administered in combination with traces of highly purified factor Xa.

Miletich and coworkers have reported specific receptor sites for factor Xa on the platelet surface and demonstrated that binding results in a substantial increase in the ability of factor Xa to activate prothrombin. As we had previously demonstrated that the thrombogenicity of certain prothrombin complex concentrates could be modified by profound thrombocytopenia, we considered the possibility that the augmentation of thrombogenicity induced by factor Xa may have resulted from its interaction with platelets. Factor V is an essential cofactor for this interaction, and in the bovine system at least, it is probably the receptor for factor Xa. As the activation of factor V appears to be essential both for optimal binding of factor Xa and a substantial increase in its procoagulant activity, we considered the possibility that the putative factor, demonstrated in our previous studies, may be a platelet-derived factor V activator similar to that suggested by the findings of Osterud and coworkers. We hypothesized that the in vivo amplification of factor Xa activity might occur following its specific binding to platelets subsequent to the activation of platelet associated factor V by the coadministration of a platelet-derived factor V activator contaminating certain products. As contamination with factor Xa is probably an inevitable consequence of the fractionation process, the final products would be more or less thrombogenic depending on the availability of the presumed factor V activator. The latter could be derived from platelets, either due to prolonged exposure of the starting plasma to blood cell components or due to irregularities in plasma separation resulting in platelet contamination of the fractionated plasma.

The studies reported here confirm the presence of a previously unsuspected thrombogenic component of certain prothrombin complex concentrates. These studies indicate, however, that the thrombogenicity of these concentrates can be attributed to phospholipids with coagulant activity rather than to an activator of factor V as initially hypothesized.

MATERIALS AND METHODS

Preparation of Prothrombin Complex Concentrates

The prothrombin complex concentrates, studied in detail in the initial studies, were made in our laboratories by a modification of the method of Cassilas and coworkers, including the modification...
described by Deggeller and co-workers. This method is currently employed by a number of agencies supplying clinically available products. Further testing included such products (see below). Great care was taken to process each plasma to an identical protocol as follows: Cohn fraction 1 of the starting plasma was prepared by adding ethanol (95% v/v) to precooled plasma (0°C) to a final concentration of 8% ethanol. The precipitate formed was separated by centrifugation at 4500 g at 0°C for 30 min. The supernatant was mixed with DEAE cellulose (Whatman DE32) and stirred for 30 min at 4°C. The slurry was separated by centrifugation at 3000 g for 30 min at 4°C and the supernatant discarded. The slurry was washed first with distilled water and then 3 times with 0.03 M sodium citrate (pH 6.8). The prothrombin complex was eluted with 0.5 M sodium chloride in 0.5 M Na2HPO4-HCl (pH 7.0). The cellulose was removed by centrifugation at 3000 g at 4°C for 30 min. The supernatant was dialyzed against large volumes of distilled water for 24 hr, after which the product was lyophilized and stored at 4°C until reconstituted for testing as described below.

Concentrates were made from two types of starting plasma. Fresh plasma was obtained by standard blood banking techniques from blood obtained from normal volunteer blood donors. The plasma was separated within 1 hr of donation and rapidly frozen and stored at −70°C. The prothrombin complex concentrate prepared from this plasma is identified as preparation “A” in the text.

The second concentrate was prepared from the supernatant plasma obtained from outdated platelet concentrates. These were obtained from the Canadian Red Cross Blood Transfusion Service, having become outdated for clinical use after 72-hr storage at 22°C with continuous gentle agitation. The platelet concentrates were pooled and centrifuged at 2500 g at 22°C for 15 min. The platelet-poor plasma was removed, snap frozen, and stored at −70°C. The prothrombin complex concentrate prepared from this plasma is identified as preparation “B” in the text.

In additional studies, comparisons of the content of coagulant-active phospholipid measured in vitro and thrombogenicity in vivo were made on a number of prothrombin complex concentrate preparations obtained from various agencies. All were considered “nonactivated” by the respective manufacturer. Two products were prepared by the methods described above. All preparations were lyophilized and reconstituted according to the manufacturer’s instructions.

Ethanol-Ether Extraction of Prothrombin Complex Concentrates

Lipid extraction was performed by adding 10 volumes of ethanol-ether (1:1) to 1 volume of the stock solution of the concentrate. The product was separated by centrifugation at 20,000 g for 10 min and the supernatant removed. The organic solvent was evaporated under a stream of pure nitrogen and the residue resuspended in 0.02 M Tris-HCl and 0.15 M NaCl (pH 7.4). The final volume was twice the volume of the original prothrombin complex concentrate.

Preparation of Purified Clotting Factors

Factor X was isolated from bovine plasma by the method of Bajaj and Mann and activated by a modification of the procedure of Downing et al. using electrophoretically homogeneous factor X activator from Russell’s viper venom (supplied by Dr. W. Kiesiel, University of Washington, Seattle) immobilized on cyanoan bromide activated agarose. Factor Xa was assayed by chromogenic assay as described later. The specific activity was 1.34 × 10⁶ U/mole (243 U/mg) factor Xa.

Prothrombin was prepared from bovine plasma by the method of Bajaj and Mans and thrombin by the method of Lundblad et al. Bovine factor V was prepared according to the modified method of Nesheim et al. and activated by thrombin as required. The preparation of DAPA was as previously described. Phosphatidylcholine-phosphatidylserine lipid vesicles (PCPS) were prepared by the method of Barenholz et al. modified as described by Nesheim et al. The single-compartment vesicles formed are of uniform dimension (325–350 Å). The final product was stored at 4°C and used within 4 days of preparation in both the in vitro and in vivo studies described. The molar ratio of phosphatidylserine to phosphatidylcholine was 1:3, based on the relative amounts of these lipids used in the preparation of the vesicles.

Quantification of Coagulant-Active Phospholipid Content

The content of coagulant-active phospholipid of each prothrombin complex concentrate was assayed in a thrombin generation assay using a synthetic inhibitor of thrombin, dansylarginine-N-(3-ethyl-1,5-pentanediyl) amide (DAPA). The assay was performed as previously described.

Qualitative Assay for Factor V Activator

A thrombin generation assay using DAPA was also used to determine the presence or absence of an activator of factor V as follows: 0.03 ml factor V (0.5 mg/ml) was incubated for 15 min at ambient temperature (22–24°C) with 0.3 ml Tris (0.02 M) NaCl (0.15 M) buffer (pH 7.4), and 0.03 ml of the test material. Of this incubation mixture 0.02 ml was then added to a mixture of factor II (1.39 × 10⁴ M), Ca²⁺ (2.0 mM), DAPA (3.0 µM), PCPS (1.7 × 10⁻⁵ M), and factor Xa (6.1 × 10⁻⁷ M) previously equilibrated at ambient temperature in the fluorometer cuvette. The rate of thrombin generation was recorded by monitoring change in intensity of fluorescence as before. Negative and positive controls were performed by substituting Tris-saline and thrombin (2 NIHu/ml, final concentration), respectively, for the test material in the primary incubation mixture. After 1-min incubation, 0.02 ml of each was transferred to a secondary incubation mixture, as described above for the test material, and thrombin generation recorded.

Characterization of Extracted Phospholipid by One-Dimensional Thin-Layer Chromatography

The phospholipids, extracted from the prothrombin complex concentrates as previously described by ethanol-ether, were identified by one-dimensional thin-layer chromatography on silica gel using two different solvent systems. Analyses were performed on 10 × 5 cm Quantigram silica gel plates (Quantum Industries, Fairfield, N.J.). The solvent systems used were chloroform:methanol:ammonia (65:25:5) and chloroform:acetonemethanol:acetic acid:water (3:4:1:1:0.5). Following chromatogram development, phospholipids were visualized with a phosphate-specific spray (Phosphry, Supelco Inc., Bellefonte, Pa.). Phospholipid components were identified by comparison with standards, including phosphatidylcholine, phosphatidylserine, phosphatidyethanolamine, phosphatidylinositol, and phosphatidic acid (Supelco Inc., Bellefonte, Pa). The identity of the lipids in the test material was determined by comparison of Rf values.

Factor Xa Assay

The factor Xa content of the prothrombin complex concentrates and the purified factor Xa was assayed by the method of Suomela et al. modified as previously described, employing the chromogenic substrate Bz-Ile-Glu-Gly-Arg-p-nitroanilide (S-2222) (Kabi-Vitrum, Montreal, Quebec) at a final concentration of 0.8 mM. A Russel viper venom (RVV) activated normal pool plasma was used as a reference standard. Arbitrarily, each milliliter of RVV-
activated normal pool was considered to contain 1 U of factor Xa activity. The same normal pool plasma was used throughout.

In Vivo Stasis Model in Rabbits

The in vivo model used was the venous stasis model described by Wessler et al.,24 modified as previously described. Male New Zealand white rabbits of between 2 and 3 kg body weight were used throughout. The animals were lightly anesthetized with sodium pentobarbital i.v. (6.2 g/dl solution, 0.5 ml/kg body weight). The test material was administered as a rapid bolus through an indwelling carotid arterial cannula and stasis induced in the contralateral jugular vein precisely 30 sec later. Fifteen minutes after stasis induction, the venous segment was opened and a thrombus reported as being either present or absent.

The dose of each prothrombin complex concentrate was administered on a dose/kg body weight basis. In the detailed comparison of concentrates “A” and “B,” the thrombogenicity of a particular dose for each concentrate was determined in groups of six animals together with negative and positive controls. Thrombogenicity was expressed as the number of animals with thrombi as a percentage of the total group tested. The threshold thrombogenic dose was defined as that dose that produced thrombi in some but not all animals tested. The thrombogenic index was defined as the reciprocal of the threshold thrombogenic dose in milliliters of stock solution per kilogram body weight administered.

Statistical Analysis

The results of the Xa assay were tested by an unpaired Student’s-t test. In comparing the coagulant-active phospholipid content of different prothrombin complex concentrate preparations with subsequent in vivo thrombogenicity, a scatter diagram was constructed and the Pearson correlation coefficient derived. The lines of best fit (see Figs. 2 and 3) were derived by least squares regression analysis.

RESULTS

Determination of In Vivo Thrombogenicity of Prothrombin Complex Concentrates “A” and “B”

Prothrombin concentrates were made as described in Materials and Methods. Preparation “A” was made from fresh frozen plasma and “B” from plasma separated from platelet concentrates that had been maintained at 22°C for 72 hr with continuous gentle agitation.

The Xa content of the stock solutions of “A” and “B” was 0.25 ± 0.03 and 0.15 ± 0.02 U/ml (mean ± SD), respectively. The difference was statistically significant (p < 0.05). In contrast, in vivo, the concentrate prepared from platelet concentrate supernatant plasma, “B,” was more thrombogenic than that prepared from fresh frozen plasma despite the latter having a higher content of factor Xa. The threshold thrombogenic dose for preparation “A” was 0.8 ml of the stock solution compared with 0.2 ml of the stock solution of preparation “B.” The thrombogenic index was consequently 1.25 for “A” and 5.00 for “B.”

Qualitative and Quantitative Assay of Coagulant-Active Phospholipid Content of Prothrombin Complex Concentrates “A” and “B”

Both concentrates were found to contain significant amounts of coagulant-active phospholipid using the thrombin generation assay employing DAPA. The results obtained are shown in Fig. 1. In each case, DAPA (3.0 μM) was incubated at ambient temperature (22-24°C) with 15 μl of the stock solution of each concentrate prothrombin (1.39 x 10⁻⁶ M) and calcium chloride (2.00 mM) in the fluorometer cuvette. A baseline recording of fluorescence was obtained and then factor Va (5.05 x 10⁻⁹ M) added and the recording continued. There was insignificant change in the intensity of fluorescence noted in the case of either test material, i.e., concentrate “A” or “B.” Factor Xa (6.06 x 10⁻⁹ M) was then added and the recording continued. The result obtained with
concentrate "A" (starting material, fresh frozen plasma) is shown in Fig. 1A and that obtained with concentrate "B" (starting material, platelet concentrate supernatant plasma) in Fig. 1B. In both cases, significant thrombin generation occurred following the addition of factor Xa. Under the conditions of enzyme (factor Xa), substrate (prothrombin) and cofactor (factor Va) concentrations used, no detectable alteration of the intensity of fluorescence would have been expected in the absence of added phospholipid. Consequently, the results suggest that both prothrombin complex concentrates contained significant phospholipid replacing activity. This activity was quantified for each preparation by reference to a standard curve constructed as previously described. The PCPS replacing activity of concentrates "A" and "B" was 2.3 x 10^-5 and 10.7 x 10^-5 moles PCPS/liter, respectively. Thus, the ratio of PCPS replacing activity for concentrate "A" compared to concentrate "B" (0.22) was similar to the ratio of their indices of thrombogenicity (0.25).

Exclusion of the Presence of a Significant Factor V Activator

As factor Va is at least 400 times more potent than factor V as a cofactor for prothrombin activation, the presence of a factor V activator may be determined by monitoring the changes in thrombin generation in an assay system where nonactivated factor V is preincubated with the test material prior to its inclusion in an incubation mixture where all other components of the prothrombinase complex are present in excess. Inclusion of DAPA ensures that feedback activation of factor V by the thrombin generated may be excluded as the effector of any activation noted.

The results of the factor V activator assays are shown in Table 1. Each of the two prothrombin complex concentrates, "A" and "B," were preincubated for 15 min with nonactivated factor V. The results shown are the amounts of thrombin generated following the addition of this preincubation mixture to secondary incubation mixtures, each containing all other components of the prothrombinase complex, with the exception of factor Va, at saturating levels. Consequently, they reflect the activation of factor V achieved during the primary incubation period. It can be seen that preincubation of factor V with either concentrate "A" or "B" did not significantly alter the rate of thrombin generation achieved as compared with the preincubation of factor V with buffer alone. In contrast, addition of thrombin to each mixture resulted in a significant increase in thrombin generation similar to that achieved when factor V alone was incubated with thrombin prior to its inclusion in the secondary incubation mixture. These results suggest that neither concentrate contained a significant activator of factor V.

Characterization of Lipid Extract of Prothrombin Complex Concentrates "A" and "B"

Each concentrate was extracted with ethanol-ether as described in Materials and Methods. The suspended extracted residue was recharacterized both in vitro by the DAPA assay and thin-layer chromatography and in vivo in the stasis model in rabbits.

Thrombin generation in assay with DAPA. In the case of both concentrates, the PCPS replacing activity was fully extractable with no activity remaining in the aqueous phase. No factor Xa, factor V/Va, or factor Va activator activity could be detected in either case.

Thin-layer chromatography. In the case of both concentrates, materials were observed that comigrated, in the solvent systems used, with authentic phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. In addition, there were components that could not be identified on the basis of the

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<th>Incubation Mixture</th>
<th>Thrombin generation (Moles/min/Liter x 10^-2)</th>
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<td>Following Addition of Incubation Mixture to Reaction Mixture</td>
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<tr>
<td>F.V + Tris saline (15 min)</td>
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<tr>
<td>F.V + PCC &quot;A&quot; (15 min)</td>
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<tr>
<td>F.V + PCC &quot;A&quot; (15 min) + thrombin (1 min)*</td>
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<td>F.V + PCC &quot;B&quot; (15 min) + thrombin (1 min)*</td>
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<td>F.V + thrombin (1 min)</td>
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*Thrombin (2 NIH U/ml) added to mixture of PCC "A"/"B" and F.V after 15-min preincubation.

Twenty microliters of incubation mixture added to reaction mixture at time (min) shown and thrombin generation determined fluorometrically with DAPA as described.

Incubation mixture: 0.03 F.V (0.5 mg/ml); 0.3 ml Tris-saline (pH 7.4); 0.03 ml PCC (neat) or Tris-saline.

Reaction mixture: prothrombin (1.39 μM); PCPS (1.7 x 10^-7 M); Xa (6.10 x 10^-6 M); Ca^{2+} (2.0 mM); DAPA (3.0 μM)—all concentrations final.
The material described in the stasis model as previously determined in the stasis model as previously described. The material was found to be nonthrombogenic when infused alone at a dose of $10^{-8}$ mole (PCPS equivalents)/kg body weight, but became thrombogenic following the addition of a purified factor Xa preparation $(3.6 \times 10^{12}$ mole/kg), prior to infusion. This dose of factor Xa was not thrombogenic when given alone to control animals.

**Correlation of Coagulant-Active Phospholipid Content With In Vivo Thrombogenicity**

The coagulant-active phospholipid content of nine different prothrombin complex concentrate preparations was determined as previously described and compared with their thrombogenic potential in vivo in the stasis model in rabbits. Five of the concentrates were prepared in our own laboratory from differing plasma sources as described in Materials and Methods. The four remaining preparations were obtained from different agencies and are all currently in clinical use in North America and Europe. The results obtained are shown in Fig. 2. The coagulant-active phospholipid content is shown as PCPS equivalents. The thrombogenic index was calculated as the reciprocal of the threshold thrombogenic dose in vivo (ml stock solution/kg body weight) determined as previously described. The correlation was highly significant ($p < 0.01$). In contrast, there was no correlation between the factor Xa content of the preparations and their thrombogenic potential determined in vivo (Fig. 3). The factor Xa content of one preparation could not be determined due to the presence of heparin added by the manufacturer.

**In Vivo Thrombogenicity of PCPS Lipid Vesicles in Combination With Factor Xa**

The thrombogenicity of PCPS vesicles with or without added factor Xa was determined in the stasis model in rabbits. When given alone, PCPS vesicles at doses as high as $8 \times 10^{-8}$ mole/kg body weight were nonthrombogenic when infused alone. In contrast, a 20-fold smaller dose $(4 \times 10^{-6}$ mole/kg) became thrombogenic when combined with a dose of purified factor Xa $(1.1 \times 10^{12}$ mole/kg). This dose of factor Xa was nonthrombogenic when infused alone in control animals.

**DISCUSSION**

The data reported in this manuscript clearly demonstrate that the thrombogenicity of some prothrombin complex concentrates is mediated by a combination of coagulant-active phospholipid and activated clotting
factors. The unique properties of DAPA and the availability of highly purified components of the prothrombinase complex enabled us to specifically identify and quantify the content of coagulant-active phospholipid in a number of prothrombin complex concentrates. There was a significant correlation between coagulant-active phospholipid content and thrombogenicity measured in vivo in a stasis model in rabbits. In contrast, no correlation was demonstrated between the content of factor Xa and thrombogenicity in vivo, suggesting that the coagulant-active phospholipid content is the more critical determinant.

The coagulant-active phospholipid extracted from the parent product retained its activity in vivo but only when combined with a purified preparation of factor Xa at a dose that was not itself thrombogenic. Thin-layer chromatography of the extracted material confirmed the presence of phospholipids with known coagulant activity. Substitution of a similar combination of vesicles of purified phosphatidylcholine-phosphatidylserine for the extracted phospholipids resulted in identical activity in vivo when combined with a nonthrombogenic dose of purified factor Xa.

In our previous studies, concentrates prepared by fractionation of plasma obtained from outdated platelet concentrates were more thrombogenic than those prepared from fresh frozen platelet-free plasma. Plasma obtained from outdated platelet concentrates was not thrombogenic in vivo when infused alone but became thrombogenic when combined with a nonthrombogenic dose of factor Xa. Consequently, we propose that the thrombogenicity of prothrombin complex concentrates is mediated by contamination of the starting plasma by coagulant-active phospholipid acting in concert with activated clotting factors generated during the fractionation procedure. As the latter appears to be an inevitable consequence of fractionation, the presence or absence of coagulant-active phospholipid assumes considerable importance in terms of potential thrombogenicity in vivo. We believe that it may be derived from either prolonged exposure of the starting plasma to blood cellular components or irregularities in separation techniques resulting in contamination of the source plasma by cellular debris. Hemolysis during blood collection, for example, may contribute substantial amounts of coagulant-active phospholipid as the inner layer of the erythrocyte membrane is a relatively rich source of phosphatidyserine.

These findings and conclusions are consistent with studies by Barton and coworkers, who demonstrated the thrombogenic potential of phospholipid in association with factor Xa. Both cephalin and disrupted platelets were shown to augment the thrombogenicity of factor Xa measured in vivo in a stasis model similar to that used in our studies. Prowse and coworkers have previously demonstrated the presence of coagulant-active phospholipid in a number of prothrombin complex concentrates. Their studies, however, were directed at assessing the correlation between phospholipid content of individual products and the degree of activation of the component clotting factors occurring during fractionation. No attempt was made to correlate coagulant-active phospholipid content with thrombogenicity in vivo.

While our data, both with the extracted phospholipid and synthetic phospholipid vesicles, were derived from studies with factor Xa, it is possible that a similar effect of phospholipid augmentation could occur with factors IXa and VII(a) in view of the formal similarity of the enzymatic complex formed from these respective clotting factors, phospholipid and calcium.

We can only speculate as to the mechanism of the apparent facilitatory role played by platelets suggested by our previous studies. Although the studies reported here clearly excluded the presence of an activator of factor V in the concentrates tested, it is possible that the confusion of coagulant-active phospholipid and factor Xa generates sufficient thrombin in vivo to trigger further platelet-associated factor V activation and the enormous potentiation of prothrombinase activity that ensues.

Although care should be exercised in extrapolating these results, obtained in vivo in an animal model to man, we believe that coagulant-active phospholipid should be considered as a major determinant of the potential thrombogenicity of prothrombin complex concentrates intended for clinical use. Moreover, the role of coagulant-active phospholipid should be evaluated in the reported factor VIII bypassing activity that these products have occasionally demonstrated in the treatment of patients with acquired inhibitors of factor VIII.

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