Anticoagulant and Fibrinolytic Activities Are Promoted, Not Retarded, In Vivo After Thrombin Generation in the Presence of a Monoclonal Antibody That Inhibits Activation of Protein C


This study examines the assumption that both the anticoagulant and fibrinolytic activity that follow the generation of thrombin induced by infusion of factor Xa/PCPS are due to generation of activated protein C. Untreated controls or animals given unrelated antibody were compared with animals pretreated with a specific monoclonal antibody to protein C (HPC4). Compared with untreated controls excess HPC4 substantially reduced the level of protein C activation as observed by protein C immunoblotting and enzyme-linked immunosorbent assay for antitrypsin/activated protein C complexes. Despite this, the anticoagulant activity as reflected by the decline of factors Va and Vllla levels (as observed by coagulation assays and by factor V immunoblotting) was significantly greater than controls. The fibrinolytic activity (as observed by assays of tissue plasminogen activator, D-Dimer, α2-antiplasmin) also was significantly greater than controls. We conclude that neutralization of the protein C anticoagulant system while resulting in a significantly more intense coagulant response to Xa/PCPS does not preclude inactivation of factors Va and Vllla and the full expression of the fibrinolytic response. We conclude further that after thrombin generation in vivo, protein C activation is not a prerequisite for the promotion of the fibrinolytic response previously observed, and that the inactivation of factors Va/Vllla may be mediated by enzymes other than activated protein C. The reduction in α2-antiplasmin levels in association with increased tissue plasminogen activator activity suggests that plasmin is a likely candidate.

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HEMOSTATIC RESPONSE TO FXa/PCPS AND ANTI-PROTEIN C

Dyserine (PCPS) vesicles were prepared as previously described by the method of Barenholz et al.54 as modified by Bloom et al.55 Type III phosphatidyicholine (egg yolk) and phosphatidylserine (bovine brain) were obtained from Sigma Chemical Co (St Louis, MO). The ratio of PCPS was 3:1. Factor V was prepared from human plasma as previously described.56 Human protein C was purified as described by Bajaj et al.57 as modified by us. Purified α-1 antitrypsin was purchased from Calbiochem (La Jolla, CA). Danyl-L-glutamylglycyl-L-arginine Chloromethyl Ketone (Dans-ECR-CK), D-Phenylnalanyl-D-Phenylnalanyl-L-Arginine Chloromethyl Ketone (Phe-Phe-Arg-CCK), and D-Phenylnalanyl-L-Prolyl-L-Arginine Chloromethyl Ketone (PAPC) were purchased from Calbiochem and reconstituted to 10 mmol/L in 1 mmol/L HCl. Immobilization membrane was purchased from Millipore Corp (Bedford, MA). Falcon PVC microliter plates were obtained from Becton Dickinson (Lincoln Park, NJ). Iodobeads were purchased from Pierce Chemicals (Rockford, IL). Bovine serum albumin (BSA) tissue culture grade was obtained from Fisher-Biotech (Fairlawn, NJ). All other chemicals used were of analytical grade or better.

Antibodies. Supplies of the murine anti-human protein C MoAb HPC4 were kindly donated by Dr Charles Esmon (Oklahoma Medical Research Foundation). The irrelevant antibody used was murine nonimmune IgG and was obtained from Calbiochem. Monospecific sheep polyclonal antibodies raised against human protein C and factor V were obtained from Affinity Biologicals (Yarker, Ontario, Canada). Specificity of each antibody was confirmed by immunoelectrophoresis against normal plasma and the purified antigens, ie, factor V and protein C.

Methods and Procedure.

Assays. Blood samples for activity based assays were anticoagulated with 1/10 vol of buffered citrate (0.06 mol/L sodium citrate, 0.04 mol/L citric acid). For immunoblotting and enzyme-linked immunosorbent assay (ELISA) studies whole blood was collected into vacuum containers containing heparin (EDTA), PPACK, and trasylol to give 1:150 dilution of 4.5 mmol/L, 20 mmol/L, 150 KIU/mL, respectively. Platelet-poor plasma was prepared immediately by centrifugation at 2,500g for 30 minutes and the plasma samples frozen at −80°C were thawed immediately before assay. Factors Va and VIIIa were measured by one-stage, and factor V by the two-stage assay as previously described.58 Fibrinogen was assayed by the method of Claus.59 α-2 antiplasmin was assayed chromogenically using the chromogenic substrate S-2251 according to the method of Eddy et al.60 Tissue plasminogen activator (t-PA) was measured functionally by the method of Verheijen et al.61 and antigenically by the method of Holvoet et al2 using the international t-PA standard kindly provided by Dr P. Gaffney (National Institute of Biological Standards, London, UK). Using urokinase, the functional t-PA inhibitor was measured by the method of Verheijen et al62 and d-dimer was measured by ELISA using the Dimer test (American Diagnostica Inc, Greenwich, CT). α-1 antitrypsin/activated protein C (α1-AT/APC) complexes were quantified by the method of Hoogenraad et al.63 Serum tumor necrosis factor levels were determined by L929 cell cytotoxicity.7,26

Electrophoresis and immunoblotting. In the case of protein C immunoblotting, non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed as described by Davis,65 using 5% acrylamide in 1.5-mm thick slabs with a 3% stacking gel. Plasma samples were diluted 1:3 in sample buffer containing 20 mmol/L EDTA and a 4-μL aliquot applied to each well. Electrophoresis was performed initially at 60 V for 30 minutes and then 150 V to completion. In the case of factor V, immunoblotting sodium dodecyl sulfate (SDS-PAGE) was performed according to the method of Neville66 using 3% to 12% acrylamide gradient in 1.5-mm thick slabs with a 3% stacking gel. Plasma samples were diluted 1:5 in sample buffers containing 5% SDS and 5% β-mercaptoethanol (B-ME) incubated at 90°C for 5 minutes followed by application of a 3-μL aliquot to each well. Electrophoresis was performed initially at 60 V for 30 minutes and then 150 V to completion. Molecular weight standards (BioRad, San Diego, CA) were run and stained with Coomassie Blue. In both cases, protein transfer to immobilization membrane was performed using a transphor unit (Pharmacia LKB Biotechnology, Uppsala, Sweden) for 2 hours at 4°C in 25 mmol/L TRIS, 192 mmol/L glycine, and 20% vol/vol methanol, pH 8.3. Immunostaining of sodium membranes was performed by the antigen overlay technique of Lamme et al67 with the following modifications. Membranes were blocked overnight at 4°C in a solution of phosphate-buffered saline (PBS) (0.015 mol/L NaCl, 0.14 mol/L Na2HPO4, pH 7.4, 0.14 mol/L NaCl, 0.002 mmol/L Na2HPO4 containing BSA (50 mg/mL) and PAPC (1 μmol/L) (blocking buffer). The blocked membranes were incubated for 3 hours at 22°C with either sheep antihuman protein C (25 μg/mL), or antihuman factor V (8 μg/mL) in PBS containing 50 mg/mL Carnation powdered skim milk (Carnation Inc, Toronto, Ontario, Canada). 0.1% Tween 20 (vol/vol), and PAPC (1 μmol/L; pH 6.5) (probing buffer). Membranes were washed for three 10-minute intervals with PBS with 0.1% Tween-20 and incubated for 1 hour with 35S-labeled human protein C or factor V diluted to 500,000 cpn/mL in the probing buffer. After further 10-minute washes, the blots were exposed to Kodak XAR-5 film with Lynex intensifying screens (Eastman Kodak Co, Rochester, NY) at −70°C. The human Protein C and factor V used in the blotting studies were iodinated to a specific radioactivity of 10 μCi/μg using Iodobeads as per manufacturer’s instructions. The mixture was then removed from the beads and gel filtered at 22°C on a 0.7 × 18 cm column of Sephadex G-25 (Pharmacia LKB Biotechnology) in tris-buffered saline containing 2 mg/mL BSA. The peaks obtained in the excluded volumes were pooled and used within 24 hours.

Animal model. Experiments were performed on seven male, juvenile baboons (Papio cynocephalus), each with a hematocrit exceeding 36% and free from tuberculosis (Table 1). Baboons were fasted overnight before each experiment and given water ad libitum. Each animal was sedated with Ketamine hydrochloride (14 mg/kg, IM) (Aveco Co Inc, Fort Dodge, IA) on the morning of the study and anesthetized with sodium pentobarbital (2 mg/kg) via a percutaneous catheter positioned in the cephalic vein. Animals were intubated orally and allowed to breathe spontaneously. The femoral artery and vein were cannulated aseptically and used for measuring arterial pressure and obtaining blood samples, respectively. The percutaneous catheter in the cephalic vein was used for infusing saline, factor Xa/PCPS and/or antibodies, and for fluid and anesthetic administration as previously described.62 Each

| Table 1. |
|-----------------|---------|----------|----------|----------|
|                | Sex    | Weight (kg) | Dose Xa (pmol/L/Kg) | Dose PCPS (pmol/L/Kg) | Dose HPC (mg/Kg) |
| Xa/PCPS (low dose) | M | 6.6 | 24.5 | 37.7 | — |
| Xa/PCPS (high dose) | M | 5.6 | 36.6 | 56.3 | — |
| Xa/PCPS (high dose) | F | 5.5 | 36.6 | 56.3 | — |
| Xa/PCPS (high dose) | F | 6.4 | 36.6 | 56.3 | 10 (non-immune IgG) |
| Xa/PCPS (high dose) | M | 5.9 | 36.6 | 56.3 | 10 |
| Xa/PCPS (high dose) | M | 4.1 | 36.6 | 56.3 | 10 |
baboon was placed on its side in contact with a controlled temperature heating pad. A light level of surgical anesthesia was maintained for the 90-minute observation period (2 mg/kg sodium pentobarbital approximately every 20 minutes).

Mean systemic arterial pressure and heart rate were monitored with a Statham pressure transducer (PD23Db; Hatoerey, Puerto Rico) and Hewlett-Packard recorder. Rectal temperature was measured with a Telethermometer (Yellow Springs Instrument Co, Yellow Springs, OH). Blood sampling was obtained via a femoral cannula. Samples were obtained at 45, 30, and 5 minutes before the infusion of factor Xa/PCPS and 2, 5, 10, 15, 20, 45, 60, and 90 minutes postinfusion. The study protocol used received prior approval by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation.

RESULTS

In initial studies, two dosage combinations of factor Xa/PCPS were evaluated with and without the preinfusion of the protein C MoAb (HPC4). The higher dose, administered per kilogram body weight, was 36.60 pmol factor Xa in combination with 56.30 nmol phosphatidylcholine/phosphatidylserine (Table 1, bottom). The lower dose was 24.50 pmol factor Xa in combination with 37.70 nmol phosphatidylcholine/phosphatidylserine (Table 1, top). In previous in vivo studies in chimpanzees, the higher dose was associated with substantially greater levels of protein C activation and fibrinolytic stimulation.14,15

Influence of Anti-Protein C MoAb on Protein C Activation After the Infusion of Factor Xa/PCPS

The activation of protein C by the infusion of factor Xa/PCPS in the presence or absence of circulating murine antihuman protein C MoAb (HPC4) was monitored by protein C immunoblotting and by quantification of α1-antitrypsin/activated protein C (α1-AT/APC) complexes by ELISA.

The results of the immunoblotting studies are shown in Fig 1. The upper panel (A) and the lower panel (B) are the results obtained using the high-dose formulation of factor Xa/PCPS in the absence or presence of HPC4 antibody (5 mg/kg), respectively. Protein C (PC) can be identified in all samples. In the animal receiving HPC4 antibody (panel B), a heavily staining band with much lower mobility is apparent. This is free HPC4 antibody, which is recognized because of the sandwich blotting technique used where the radiolabeled protein C antigen is used for detection. It can be seen that excess HPC4 antibody persists throughout the course of observation of the treated animal. In the case of the HPC4 antibody untreated animal (panel A) immediately after the factor Xa/PCPS infusion, a band (A) with lower mobility than protein C appears. This increases in density over time but is fading by 90 minutes. This corresponds to protein C in complex with protein C inhibitor (PCI)14. A second band (B) of faster mobility also appears. This reaches peak density by 15 minutes and, unlike A, is maintained over the balance of the period of observation. This corresponds to activated protein C in complex with α1-antitrypsin (α1-AT).14 In contrast, the animal pretreated with HPC4 shows no evidence of formation of these activated protein C/ inhibitor complexes.

The α1-AT/APC complexes monitored qualitatively by immunoblotting were quantified by ELISA and the results are shown in Fig 2. The quantity of complex generated over time in the animals receiving high-dose factor Xa/PCPS plus HPC4 antibody was compared with that generated in control animals receiving factor Xa/PCPS plus either saline or irrelevant murine antibody. The conditions of both studies including the time and dosage of Ig were identical. Infusion of HPC4 antibody and factor Xa/PCPS is associated with a substantial reduction in the level of α1-AT/APC complexes generated in comparison with the animals that were infused with either saline or the irrelevant antibody plus factor Xa/PCPS.
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Influence of Anti-Protein C MoAb on the Functional Activity of Factors V/Va and VIIia After Infusion of Factor Xa/PCPS

Factors Vα and VIIIα were measured by the one-stage and total factor V by the two-stage clotting assay. In the two-stage clotting assay, the plasma sample was preincubated with thrombin before performing a one-stage assay. This maneuver activates the cofactor. Therefore, the result obtained in the two-stage assay is considered to represent the total amount of the cofactor available. Conversely, the one-stage assay result is considered to represent the activated fraction of each cofactor in the sample collected.

The results obtained with the high dose of factor Xa/PCPS are shown in Fig 3 (factor V/Va) and Fig 4 (factor VIIIα). Comparisons were made between the responses of factors Vα and V in animals receiving an infusion of factor Xa/PCPS in the absence (control) and presence of HPCα antibody. Figure 3A shows that infusion of factor Xa/PCPS alone is followed by a sustained reduction in factor Vα activity to approximately 65% of the T-0 value by 15 minutes. In contrast, preinfusion of HPCα antibody reduced this factor Vα activity to 38% of the T-0 value by 3 minutes. Figure 3B shows that the infusion of factor Xa/PCPS alone is followed by a transient reduction of total factor V activity to 75% of the T-0 value at 15 minutes, after which point total activity returned to 90% of the T-0 value. Because the error of the factor V assay is ±10%, it cannot be said that this trend is real. In contrast, preinfusion of the HPCα antibody reduces this factor V activity to 25% of the T-0 value at 3 minutes. Using the lower dose of factor Xa/PCPS, although the effects were less marked, the trends with regard to the influence of pretreatment with HPCα were similar (results not shown). Figure 4 shows that the loss of factor VIIIα activity like that of factor Vα is substantially greater in the presence of HPCα antibody than in its absence.

In summary, there is loss of factor Vα and factor VIIIα with minimal loss of factor V total activity after infusion of factor Xa/PCPS alone, whereas preinfusion of HPCα antibody permits a profound and rapid loss of activity of factor V as well as factors Vα, VIIIα. This is not what would be predicted if inactivation of factors Vα and VIIIα were due solely to generation of activated protein C shown in Figs 1 and 2.

**Fig 2.** The amount of APC in complex with α1-antitrypsin (α1-AT) was quantified by ELISA as described in Materials and Methods. The quantity of α1-antitrypsin/activated protein C complexes (α1-AT/ APC) generated in five animals over time (minutes) after the infusion of factor Xa/PCPS (●) alone (Δ, △, ◼, ◼, ◼, ◼, Table 1), factor Xa/PCPS plus HPCα antibody (●, ○, ○) (animals no. 4, 8, Table 1), and factor Xa/PCPS plus irrelevant antibody (●, ◻) (animal no. 9, Table 1), are shown. The timing of the infusion of HPCα antibody where given is indicated by (●).

**Fig 3.** The change in factor Vα, factor V coagulant activity as a function of time (minutes) after the infusion of high-dose factor Xa/PCPS (●) either alone (Δ, △) (animal no. 3, Table 1), or plus HPCα antibody (●, ○) (animal no. 4, Table 1) are shown. The timing of HPCα antibody infusion where given is indicated by (●). The results of assays of plasma from the alternate pair of animals were similar to those shown in this graph.

**Fig 4.** The changes in factor VIIIα response of factor Vα and V coagulant activity as a function of time (minutes) after the infusion of high-dose factor Xa/PCPS (●) either alone (Δ, △) (animal no. 3, Table 1), or plus HPCα antibody (●, ○) (animal no. 4, Table 1), are shown. The timing of HPCα antibody infusion where given is indicated by (●). The results of assays of plasma from the alternate pair of animals were similar to those shown in this graph.
Influence of Anti-Protein C MoAb on Proteolysis of Factor V After Infusion of Factor Xa/PCPS

The changes induced in factor V also were followed by immunoblotting of duplicate samples collected into the anticoagulant cocktail designed to prevent the in vitro proteolysis described in Materials and Methods. These were subjected to SDS-PAGE and immunoblotting. The results obtained are shown in Fig 5. One animal received factor Xa/PCPS; the other received factor Xa/PCPS at the same dose plus HPC$_i$ antibody. In both cases, samples obtained 5 minutes before the infusion of factor Xa/PCPS show a major band with an apparent molecular weight of 283 Kd, which is factor V. Minor low molecular weight bands (87 and 53 Kd apparent) are seen at equivalent concentrations in both animals and persist throughout. Two minutes after the infusion of factor Xa/PCPS significant fragmentation of factor V is apparent in both series. However, in the case of the HPC$_i$ antibody-treated animal there is a substantial reduction in the density of the 283-Kd band. This is consistent with the decrease in total factor V noted by coagulation assay. This reduction of the 283-Kd band coincides with the appearance of new bands 260, 194, and 179 Kd. The generation of these three fragments is amplified by preinfusion with HPC$_i$ antibody. In addition, changes unique to animals infused with HPC$_i$ antibody are also observed. First, a band at 139 Kd seen in the control disappears in the animal receiving HPC$_i$ antibody, coincident with the appearance of the 126-Kd band. Second, a 29-Kd band appears in the animals receiving HPC$_i$ antibody, which is absent in the controls.

Interpretation of these data is limited by lack of information on the fragmentation pattern of baboon factor V after enzymatic treatment with thrombin, factor Xa, etc. However, the evidence presented demonstrates that although the precise nature of the enzymatic event is not characterized, the event itself appears to be amplified rather than attenuated by the inhibition of protein C activation. In fact, inhibition of protein C activation with the HPC$_i$ antibody results in a degradation profile that is different from that seen in absence of HPC$_i$, as reflected by the appearance of 29-Kd and 126-Kd fragments.

In summary, there is both activity and physical evidence that inhibition of protein C activation after factor Xa/PCPS infusion results in a rapid degradation of both factor Va and V and that the pattern of degradation is unique with respect to the uninhibited response to factor Xa/PCPS. These results raise the question, by what means are factors Va and V inactivated, if not by activated protein C? A related question is what is the effect of inhibition of protein C activation on the fibrinolytic response to factor Xa/PCPS? Is the fibrinolytic response to factor Xa/PCPS enhanced by inhibition of protein C activation in the same manner as the degradation of factors Va and V? Are these two events connected?

Influence of Anti-Protein C MoAb on Fibrinolytic Response After Infusion of Factor Xa/PCPS

Plasma samples obtained during the course of the experimental protocol were assayed for levels of fibrinogen, t-PA functional and antigen activity, α-2 antiplasmin, and D-Dimer.

Pretreatment with HPC$_i$ antibody before the infusion of either high- or low-dose formulations of factor Xa/PCPS was associated with substantially greater decreases in the level of fibrinogen (Fig 6). In the case of the low-dose formulation there was a relatively minor (25%) decrease in fibrinogen level over the course of the experiment. In contrast, pretreatment with antibody was associated with a 70% decrease within 15 minutes, after which it plateaued at that level. With the high-dose control there was a 30%
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The changes in fibrinogen after the infusion of factor Xa/PCPS (△), either alone (△ A) or plus HPC, (Θ-Θ) are shown. (A) The response after the low dose (animals no. 1, 2, Table 1) and (B) the high dose (animals no. 3, 4, Table 1) of factor Xa/PCPS. HPC, antibody infusion is indicated by (△). The results of assays of plasma from the alternate pair of animals were similar to those shown in this graph.

decrease by 15 minutes, whereas in the presence of HPC, antibody fibrinogen was no longer detectable by the time the first postfactor Xa/PCPS infusion sample was taken.

Figure 7 illustrates the changes in levels of fibrinogen (panel A) and D-Dimer (panel B) after the infusion of the high-dose formulation of factor Xa/PCPS in three animals: one treated with factor Xa/PCPS alone, one pretreated with HPC, antibody before factor Xa/PCPS infusion, and the third pretreated with an irrelevant murine antibody before factor Xa/PCPS infusion. The response to factor Xa/PCPS is very similar between the untreated control and that receiving the irrelevant antibody and both show substantially less change than the animal receiving HPC, antibody. Similarly, changes in D-Dimer are equivalent in the two control animals and they are substantially less than that seen in the animal receiving HPC, antibody.

Figure 8 shows the changes in t-PA functional activity (panel A), t-PA antigen level (panel B), and α-2 antiplasmin (panel C) in the same three animals described in Fig 7. Again, equivalent changes are noted in the two control animals and substantially greater increases in t-PA activity and antigen level in the HPC, antibody-treated animal. This is associated with a substantially greater reduction in the circulating levels of α-2 antiplasmin, which has reached a level 40% of the preinfusion concentration 20 minutes after the infusion of factor Xa/PCPS. These latter data suggest that not only is the release of t-PA promoted in the presence of HPC, antibody, but subsequently substantial plasmin generation occurs with the consequent loss of free α-2 antiplasmin in complex.

All plasma samples were assayed for functional plasminogen activator inhibitor (PAI-1). No inhibitor activity could be detected, suggesting that either the circulating levels were below the lower limit of the sensitivity of the assay (<6 IU/mL) or species specificity existed with regard to the recognition of human t-PA, used in the assay procedures, by baboon plasminogen activator inhibitor.
Influence of Anti-Protein C MoAb on the Response of the Cardiovascular System and Formed Elements of the Blood After Infusion of Factor Xa/PCPS

The responses of the cardiovascular system and formed elements of the blood are shown in Tables 2 and 3. Significant changes from baseline (P < .05) are indicated by the asterisks.

Infusion of factor Xa/PCPS is followed by a brief decrease in mean systemic arterial pressure (MSAP) and neutrophil count with a reciprocal increase in heart rate between 1 and 5 minutes (Table 2). The decrease in mean systemic arterial pressure is accentuated by coinfusion of HPC, with the factor Xa/PCPS (Table 3). This is accompanied by a decrease in platelet count and a decrease in hematocrit that were not seen after infusion of factor Xa/PCPS alone. These responses occur at the same time that the fibrinogen concentration reaches its nadir (5 minutes) (Fig 7) and they correct at the same time that the markers of fibrinolytic activity reach their peak (Fig 8). However, there is no evidence of change in temperature or a release of tumor necrosis factor (TNF) (data not shown).

**DISCUSSION**

Stearns et al\(^6\) and Taylor et al\(^7\) have previously demonstrated that the murine human protein C MoAb (HPC,) inhibits the activation of protein C both in vitro and in vivo. These studies confirm that the antibody is also active in preventing the activation of protein C after the infusion of factor Xa in combination with PCPS as a source of coagulant active phospholipid. This observation permitted both the inactivation of factor V and Va and the increased availability of t-PA. In the case of factor V inactivation, inhibition of protein C activation resulted in the increased cleavage of both factors Va and V. Although the pattern of cleavage of baboon factor V has not been the focus of this study, comparison with data available from studies of human, bovine, and canine factor V suggests that the pattern of fragmentation is due to some proteases other than activated protein C. In particular, comparison with human factor V cleavage patterns suggest that the presence of a 260-Kd fragment is due to factor Xa rather than thrombin.\(^6\) Moreover, the fact that factor V, which is a poor substrate for activated protein C,\(^6\) is cleaved suggests inactivation by proteases other than activated protein C. Plasma has been shown to initially activate and then rapidly inactivate factor V.\(^3,37\) In view of the clear demonstration of increased t-PA availability and increased plasmin generation reflected by a substantial reduction in the level of its principal inhibitor of α2-antiplasmin, it is reasonable to suppose that the changes noted in the presence of HPC, antibody may result from plasmin as opposed to activated protein C inactivation of factor V. This and/or nonenzymatic neutralization may also account for the changes in factor VIIIa.

In the case of increased fibrinolytic activity, inhibition of protein C activation initially would be expected to augment

<table>
<thead>
<tr>
<th>Table 2. Changes (mean ± SD) in Vital Signs and Blood Counts in Control Animals (n = 3) (no. 3, 7, and 9, Table 1) Administered Xa/PCPS</th>
<th>Elapsed Time</th>
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<td>T-0 T + 1 T + 2 T + 5 T + 10 T + 15 T + 30 T + 60 T + 90</td>
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<td><strong>MSAP mm/Hg</strong></td>
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<td><strong>HR/min</strong></td>
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<td><strong>Hct Yo</strong></td>
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<tr>
<td><strong>Pl × 10^3/L</strong></td>
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**Abbreviations:** MSAP, mean systolic arterial pressure; HR, heart rate; WBC, leukocyte count; temp, temperature; Hct, hematocrit; Resp, respiration rate; Pl, platelet count.

*Significant change over baseline (P < .05).
†Elapsed time after infusion of factor Xa/PCPS.
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Table 3. Changes (mean ± SD) in Vital Signs and Blood Counts in Test Animals (n = 3) (no. 2, 4, 8) (Table 1) Administered Xa/PCPS + HPC,

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Abbreviations: MSAP, mean systolic arterial pressure; HR, heart rate; WBC, leukocyte count; Temp, temperature; Hct, hematocrit; Resp, respiration rate; Pl, platelet count.

*Significant change over baseline (P < .05).
†Elapsed time after infusion of factor Xa/PCPS.

both factor Xa and thrombin generation due initially to the relatively unrestricted activity of the cofactors Va and VIIIa in the tenase and prothrombinase function. This initial burst of thrombin activity leads to increased plasma levels of t-PA and fibrinolytic activity. We believe that this initial effect is countered by plasmin hydrolysis of factors V and VIII and Va and VIIIa acting as a negative feedback. Monitoring of the fibrinolytic parameters clearly demonstrates that inhibition of protein C activation by HPC, antibody permits this. Therefore, this observation lends further support to previously reported studies suggesting that the increase in t-PA availability could be directly correlated to the intensity of the thrombotic stimulus and presumably the level of thrombin and/or factor Xa generated.

The abrupt decrease followed by an equally rapid return to normal blood pressure, hematocrit, and platelet count at T + 1 to T + 5 minutes coincides with generation of thrombin and consumption of fibrin followed by the appearance of tissue plasminogen activator and plasmin-antiplasmin complexes. However, the systemic effects of factor Xa/PCPS are limited to perturbation of these hemostatic parameters and vital signs. They are amplified by inhibition of the activation of protein C. We conclude that these effects and their resolution is due to the generation of thrombin followed by the release of t-PA and a fibrinolytic response. There is no associated inflammatory response such as the appearance of TNF in the plasma. However, the transient decrease in total white blood cell count may reflect a transient margination of segmented neutrophils after expression by the endothelium of receptors such as GMP-140 induced by thrombin. The prolonged depression of fibrinogen concentration as late as 90 minutes after infusion of Xa/PCPS is unexplained. Continued hydrolysis of fibrinogen as well as fibrin may account for this as t-PA and plasmin-antiplasmin complexes are still present as late as T + 90 minutes.

In summary, further evidence is presented for the presence of multiple interactions between coagulation and fibrinolytic systems. The fibrinolytic system has the potential not only to rapidly clear fibrin and, in some situations, degrade fibrinogen, but also to exercise a potent anticoagulant activity. Previously, this has been recognized in general terms given its potential to nonspecifically degrade all hemostatically important coagulant proteins. The observations presented here suggest that the anticoagulant activity of the fibrinolytic system is expressed mainly through the inactivation of the cofactors V and VIII and possibly hydrolysis of fibrinogen, which exert a highly significant regulatory role over coagulant function. Thus, the substitution of a relatively nonspecific anticoagulant influence exerted over factor V and VIII availability by plasmin for the relatively specific regulation exerted over factor Va/ VIIIa by activated protein C may have major implications in the management of bleeding disorders such as disseminated intravascular coagulation. To characterize the relative contributions that each process may make, it will be necessary to (1) develop methodologies capable of identifying specific cleavage products of factors V and VIII, if they exist, of the two inactivation pathways, and (2) intervene with inhibitors of thrombin and plasmin (ie, hirulog/ hirudin, plasmin antibodies).

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Anticoagulant and fibrinolytic activities are promoted, not retarded, in vivo after thrombin generation in the presence of a monoclonal antibody that inhibits activation of protein C

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