Activated prothrombin complex concentrate (APCC)-mediated activation of factor (F)VIII in mixtures of FVIII and APCC enhances hemostatic effectiveness

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Running title: APCC-mediated activation of FVIII

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SUMMARY

***Background and objectives*:** Activated prothrombin complex concentrates (APCC), utilized in bypassing therapy for hemophiliacs with inhibitor, contain factors (F)VII, FII, FIX and FX, and their active forms. A recent report has demonstrated that mixtures of APCC and FVIII potentiated thrombin generation, *in vitro,* in plasma from patients with severe hemophilia A, but mechanism(s) remain unknown. ***Results*:** APCC (0.05 U/ml) increased FVIII activity ~4-fold within 1 min in one-stage clotting assays, followed by a return to initial levels within 10 min. This reaction was dependent on the presence of tissue factor and phospholipid. Thrombin generation produced from APCC was ~3.5-fold greater in the presence of FVIII than that in its absence. SDS-PAGE analysis revealed that APCC sequentially proteolyzed the heavy chain of FVIII at Arg^{372} and Arg^{740}, followed by cleavage at Arg^{336}. Proteolysis was prevented by FVIIa inhibitor, but not by hirudin, supporting the concept that APCC itself possessed the potential to activate FVIII in early coagulation phases, and that FVIIa in APCC contributed mainly to this reaction. APCC-mediated FVIII activation was unaffected by the addition of anti-FVIII inhibitor antibodies, irrespective of epitope specificity. Anti-C2 type 1 inhibitors, however, diminished the inactivation phase of the APCC reaction by inhibiting cleavage at Arg^{336}. ***Conclusion*:** Small amounts of APCC, relative to the standard concentration used for clinical purposes, could activate FVIII directly, even in the presence of anti-FVIII antibodies. Combination therapy based on mixtures of APCC and FVIII could have significant beneficial implications for the treatment of hemophilia A patients with inhibitors.

Keywords: activation analysis, blood coagulation, factor IX, factor VII, factor X – prothrombin drug combination, factor VIII – human, hemophilia A, inhibitors.

INTRODUCTION

Factor (F)VIII, a plasma protein deficient in individuals with the severe congenital bleeding disorder, hemophilia A (HA), functions as a cofactor in the tenase complex, responsible for phospholipid (PL)-dependent FIXa-mediated activation of FX [1]. The FVIII molecule is arranged into three domains (A1-A2-B-A3-C1-C2), and is processed into series of heterodimers, generating a heavy chain (HCh) consisting of A1 and A2 domains together with heterogeneous proteolyzed B domain linked to a light chain (LCh) consisting of A3, C1, and C2 domains [2]. The catalytic efficiency of FVIII in the tenase complex is markedly enhanced by conversion to FVIIIa, through limited proteolysis by thrombin and FXa. Both enzymes proteolyze FVIII at Arg^{372} and Arg^{740} in the HCh, resulting in the generation of 50-kDa A1 and 40-kDa A2 subunits. The 80-kDa LCh is proteolyzed at Arg^{1689} producing a 70-kDa subunit [3]. Proteolysis at Arg^{372} and Arg^{1689} is essential for generating FVIIIa cofactor activity. Cleavage at the latter site liberates FVIII from its carrier protein, von Willebrand factor (VWF) [4]. FVIIIa activity is down-regulated by activated protein C,

following cleavage at Arg^{336} [3].

FVIII inhibitors develop as alloantibodies (alloAbs) in 20-30% of multi-transfused HA patients and also arise as autoantibodies (autoAbs) in previously normal individuals resulting in acquired HA [5]. The reduction or disappearance of FVIII activity (FVIII:C) with inhibitors is associated with impairment of FVIII(a) cofactor function mediated by binding to functionally essential regions on FVIII. FVIII inhibitors either inhibit FVIII:C completely or incompletely at saturating concentrations, corresponding to a classification of type 1 or type 2, respectively [6]. Inhibitory epitopes have been localized to one or more of the A2, A3-C1, and C2 domains [7,8]. Anti-C2 antibodies classified as type 1 prevent FVIII binding to PL and VWF, whilst those classified as type 2 prevent FVIII binding to thrombin and FXa [9-12]. Anti-A2 and anti-A3-C1 antibodies prevent FVIIIa interaction with FIXa [8,13]. "Bypassing therapy", utilizing recombinant FVIIa (rFVIIa; Novoseven®, Novo Nordisk) and plasma-derived activated prothrombin complex concentrates (APCC; FEIBA®, Baxter) are utilized for the treatment of HA patients with inhibitor classed as high responders.

We have recently demonstrated that FVIIa/TF complex rapidly activated FVIII by limited proteolysis at Arg^{372} and Arg^{740} in HCh [14]. This reaction appeared to promote a "priming" mechanism to generate small amounts of FVIIIa physiologically in the early initiation phases of the coagulation process. The potential for activation was markedly less than that mediated by thrombin, because of rapid cleavage at Arg^{336} and little cleavage at Arg^{1689}. In addition, activation was observed even in the presence of anti-FVIII antibodies, independent of inhibitor-epitope specificity and kinetics. Inactivation was moderated, however, by anti-C2 antibody with type 1 characteristics [15].

Berntorp and colleagues have recently demonstrated that mixtures of FVIII and bypassing agents (rFVIIa and APCC) compared to bypassing agents alone synergistically potentiated thrombin generation, *in vitro*, in plasma samples from HA patients with inhibitor [16]. The specific mechanism(s) has not been identified, however. In this study, we have examined the hemostatic properties of mixtures of FVIII and APCC, and have demonstrated that pre-activation of FVIII by FVIIa containing in APCC could contribute to enhanced coagulation potential. The results provide challenging implications for the future therapeutic use of combination therapy in HA patients with inhibitors.

MATERIALS AND METHODS

Reagents - Recombinant FVIII and APCC (FEIBA®) preparations were generously provided by Bayer Corp. Japan (Osaka, Japan) and Baxter Japan (Tokyo, Japan), respectively. Recombinant hirudin (Calbiochem, San Diego, CA), recombinant lipidated TF (Innovin®; Dade Behring, Marburg, Germany), E-76 peptide (Bachem, Torrance, CA), chromogenic substrate S-2222 and S-2238 (Chromogenix, Milano, Italy), goat anti-mouse peroxidase-linked secondary antibody (MP Biomedicals, Aurora, Ohio), plasma-derived FVIII-deficient plasma (George King Biomedical, Overland Park, KS) were purchased from the indicated vendors. Anti-FVIII monoclonal antibodies (mAb)C5, recognizing the A1 domain [15] (epitope 351-365) and anti-A2 mAb413 (epitope 484-509 [17]) were provided by Drs. Carol Fulcher and Evgueni Saenko, respectively. An anti-A2 mAbJR8 was obtained from JR Scientific Inc. (Woodland, CA). Anti-C2 mAbs ESH4 (epitope 2303-2332) and ESH8 (epitope 2248-2285) were purchased from American Diagnostica Inc. (Greenwich, CT) [18]. PL vesicles (phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine; 10%:60%:30%) were prepared using *N*-octylglucoside [19].

Anti-FVIII inhibitor antibodies - Six anti-FVIII inhibitor alloAbs or autoAbs were obtained from Japanese patients with congenital or acquired HA, respectively. IgG fractions were prepared using protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden). $F(ab')_2$ fragments were prepared using immobilized pepsin-Sepharose (Pierce, Rockford, IL). The inhibitor titer of antibody-$F(ab')_2$ was determined by the Bethesda assays. The kinetic patterns of FVIII inactivation by anti-FVIII antibodies (type 1 and type 2 characteristics) were determined in one-stage clotting assays. The epitope specificities of inhibitor antibodies were determined by SDS-PAGE and Western blotting using isolated FVIII fragments.

FVIII activation by APCC - All reactions were performed at 37°C in 20 mM HEPES pH 7.2, 0.1 M NaCl, and 0.01% Tween 20 (HBS) containing 5 mM $CaCl_2$ and 0.1% bovine serum albumin. FVIII (10 nM) was mixed with the indicated concentrations of APCC together with TF (0.5 nM) and PL (20 μM). Samples were obtained from the mixtures at the indicated times, and the APCC reaction was immediately terminated by the addition of FVIIa-inhibitor E-76 (2.5 μM) and thrombin-inhibitor hirudin (2.5 U/ml) and 500-fold dilution. FVIII:C was measured in one-stage clotting assays using FVIII-deficient plasma. The presence of APCC, E-76, and hirudin in the diluted samples did not affect FVIII:C measurements (<0.5%). To assess the effects of anti-FVIII inhibitor antibodies, FVIII (10 nM) was preincubated with the indicated concentrations of antibody-$F(ab')_2$ for 1 hr (showing stability of the FVIII-antibody complex). The mixtures were reacted with APCC (0.05 U/ml), PL (20 μM) and TF (0.5 nM), and the reactions were terminated by the addition of E-76 and hirudin, and 200-fold dilution. The final concentrations of inhibitors were adjusted to 2 BU/ml. Control-$F(ab')_2$

was used at a concentration of 500 nM.

Chromogenic assays for FXa and thrombin generation - FVIII (10 nM) was mixed with PL (20 μM), TF (0.5 nM), and S-2222 (FXa substrate) or S-2238 (thrombin substrate) in HBS-buffer containing 5 mM $CaCl_2$. Endogenous FXa and thrombin generations were initiated by the addition of APCC (0.05 U/ml), and were quantified by absorbance at 405 nm using a Labsystems Multiskan Multisoft® microplate reader (Helsinki, Finland). In FXa-assays, hirudin (2.5 U/ml) was added to the samples to completely exclude the feedback activation of FVIII by generated thrombin.

Cleavage of FVIII by APCC - FVIII (10 nM) was incubated with APCC (0.05 U/ml), PL (20 μM), and TF (0.5 nM) in HBS-buffer with 5 mM $CaCl_2$. Aliquots were obtained at the indicated times, and the reactions were immediately terminated by boiling for 3 min and prepared for SDS-PAGE. To examine the effects of anti-FVIII inhibitor antibodies, FVIII was preincubated with antibody-F(ab')$_2$ (200 nM) for 1 hr, prior to the addition of APCC. SDS-PAGE and Western blot was performed using 8% gels at 150 V for 1 hr [17]. Protein bands were identified using the indicated mAbs, followed by a goat anti-mouse peroxidase-linked mAb. Signals were detected using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA).

RESULTS

Activation of FVIII by APCC - To investigate the possibility that the enhanced hemostatic effects in mixtures of APCC and FVIII [16] might be related to a direct association, direct activation of FVIII by APCC was initially examined in one-stage clotting assays. FVIII (10 nM) was incubated with various concentrations of APCC with TF (0.5 nM) and PL (20 μM). APCC activity was terminated by the addition of hirudin and E-76 peptide, and higher dilution at the indicated times. The minimum level of measurement of FVIII:C in this assay was ~0.01 nM, and in these experiments, FVIII was used at 10 nM, ~10-fold higher than physiological concentrations.

In mixtures of FVIII and APCC together with TF and PL, FVIII:C rapidly reached peak levels, 2.5~9-fold of initial level, within 3 min after the addition of various concentrations of APCC (0.025-0.1 U/ml). These reactions were dose-dependent (Fig. 1A), suggesting that APCC did activate FVIII. Subsequently, the peak FVIII:C returned to initial levels after ~10 min. The addition of TF enhanced the APCC-mediated activation dose-dependently, and reactions appeared to be nearly saturable (~6-fold of initial) at 0.5 nM TF (Fig. 1B). In the absence of TF, increases of FVIII:C were not significantly observed within 10 min after the addition of APCC. The APCC-mediated activation was little observed after 30 min in the absence of PL, however (data not shown). These findings suggested that the early APCC-mediated FVIII activation depended on the

TF and PL, possibly due to the direct activities of FVIIa and/or FXa. Later FVIII activation may not have been dependent on TF, but may have been due to the generated thrombin in the reaction mixtures. Subsequent experiments were performed using the concentrations of APCC (0.05 U/ml) and TF (0.5 nM).

Effect of FVIII on thrombin formation generated from APCC itself - To further investigate that APCC-mediated activation of FVIII governed the enhancing hemostatic effect, we examined the thrombin generation from APCC itself utilizing purified reagents without FVIII-deficient plasma in chromogenic assays. Thrombin formation was initiated in APCC solutions (0.05 U/ml) with or without FVIII by the addition of mixtures of PL (20 μM), TF (0.5 nM), and thrombin substrate. In the absence of FVIII, thrombin generation was observed in a timed-dependent manner, but in its presence, the potential for thrombin generation was markedly greater (Fig. 2). The maximum generated thrombin in the presence of FVIII appeared to be ~3.5-fold higher than that in its absence at 3 min after reaction. Thrombin generation was not observed in the absence of TF, however.

Proteolytic cleavage of FVIII by APCC - The APCC contains various concentrations of at least four zymogens (prothrombin, FVII, FIX, and FX) and their active forms. The active molecules proteolyze FVIII at Arg^{336}, Arg^{372}, or Arg^{740} (in HCh), and Arg^{1689} or Arg^{1721} (in LCh) [3,14]. Cleavages at Arg^{372} and Arg^{336} are essential for activating and inactivating cofactor activity, respectively. Association of FVIII with FXa (and FIXa), FVIIa, and thrombin are PL-dependent, PL/TF-dependent, and PL/TF-independent, respectively [3,14]. To investigate whether APCC-mediated FVIII reaction was governed by proteolytic HCh cleavage during early-timed reactions, temporal changes in electrophoretic mobility of APCC-treated FVIII were examined by SDS-PAGE (Fig. 3). Products of proteolysis were visualized using an anti-A2 mAbJR8 (*panel* A) and anti-A1 mAbC5 (*panel* B). In the absence of PL (*panel* a), the intact HCh (A1-A2-whole B) was little proteolyzed within 10 min after the addition of APCC, irrespective of TF. When PL was added, the intact HCh was moderately degraded to A1-A2 fragments by cleavage at Arg^{740}, followed by the generation of A2 fragments by cleavage at Arg^{372} within 5 min, indicating that APCC-mediated proteolysis in these circumstances was initiated by PL-dependent protease(s), but not by thrombin. In the presence of PL and TF, markedly rapid cleavage at Arg^{740} and Arg^{372} was seen within 1 min. In repeated experiments with hirudin, APCC-mediated cleavage of HCh was similarly observed, although cleavage at Arg^{740} and Arg^{372} was slightly delayed, supporting the feedback mechanism of FVIII activation by generated thrombin in the presence of PL and TF (*panel* b). These results suggested that APCC-mediated activation was initiated predominantly by FXa on the PL surface in the absence of TF, but was initiated by FVIIa in its presence.

The role of A1 cleavage at Arg^{336} in APCC-mediated inactivation of FVIII(a) was further examined. Products of proteolysis at Arg^{336} were identified using an anti-A1 mAbC5 (Fig. 3B *panel* a). Band densities of $A1^{1-372}$ were assessed by quantitative densitometry and were compared at equivalent amounts of FVIIIa (*panel* b). Since this mAb recognizes the acidic region in A1, the appearance and disappearance of 50-kDa fragment ($A1^{1-372}$) represent cleavages at Arg^{372} and at Arg^{336}. In mixtures of APCC with TF and PL, the $A1^{1-372}$ appeared very rapidly within 1 min, followed by slow disappearance, suggesting rapid cleavage at Arg^{372} and slow cleavage at Arg^{336}. The addition of E-76 delayed cleavage at Arg^{372}, but did not significantly affect cleavage at Arg^{336}. The results indicated that FVIIa contributed to the APCC-mediated cleavage of HCh in a TF-dependent mechanism. In the absence of TF, the $A1^{1-372}$ appeared more slowly but disappeared slightly rapidly relative to that in its presence. The addition of E-76 had no effect in the absence of TF, however, confirming the role of FXa in TF-independent APCC-mediated proteolysis.

Direct FXa generation by APCC in the presence of FVIII - FXa generation, corresponding to the upstream process of thrombin generation, was examined to further investigation of the mechanism(s) on direct APCC-mediated activation. The FXa generated by APCC in the presence of FVIII were measured in a chromogenic assay as described in Methods (Fig. 4). FXa was not generated from APCC within 10 min in the absence of TF, but was markedly increased in a time-dependent manner in its presence, and reached a plateau after ~2 min. The E-76 was added to the mixtures prior to the reaction with APCC to again evaluate a role of FVIIa in APCC-derived FXa generation. The presence of E-76 significantly diminished FXa formation. FXa generation was little detected after 2 min but then gradually increased to reach a peak level at 10 min. Significant FXa generation was not observed without FVIII (data not shown). The results suggested that APCC-induced FXa generation in the presence of TF and FVIII in early-timed reaction phases was predominantly governed by the FVIIa activity. Additionally, FXa generation at later time points appeared to be due to FX activation caused by FVIIa that was markedly depressed by E-76.

APCC-mediated FVIII activation with anti-FVIII inhibitor antibodies - FVIIa/TF activates FVIII even in the presence of anti-FVIII inhibitor antibodies, irrespective of the epitope-specificity [15]. APCC-mediated FVIII reactions were also examined in the presence of anti-FVIII antibodies. The inhibitor titers of all antibodies (two anti-A2 type 1, three anti-C2 type 1, and one anti-C2 type 2) were adjusted to 2 BU/ml final concentration. Representative timed-course reactions are shown in Fig. 5A-a, and the ratio of peak FVIII:C relative to control are illustrated in Fig. 5A-b. Initial FVIII:C in the presence of antibodies prior to the addition of APCC was 2~3 U/dl. APCC-mediated FVIII activation was similar to that with the control-$F(ab')_2$ and was not significantly depressed by any antibodies. Similar experiments using higher inhibitor titers (>5 BU/ml) failed to evaluate the

change of FVIII:C, since the activity levels were much lower after higher dilution.

The effects of the anti-FVIII antibodies on the inactivation phase of APCC-mediated reactions were evaluated. Peak levels of FVIII:C after the addition of APCC were regarded as 100%, and the time-dependent decreases in FVIII:C were evaluated by analyzing single exponential decay [15]. Spontaneous dissociation of the A2 domain from FVIIIa might have affected measurements of the rate constants obtained in the inactivation phase, however, and the results represented were limited, therefore, to comparisons with that of control, and were expressed as apparent in these experiments. The apparent rate for control-$F(ab')_2$ was 0.34 ± 0.05 min^{-1}, and the ratios of antibody-inactivation rates relative to control are illustrated in *panel* b. Anti-A2 (cases 1 and 2) and anti-C2 type 2 (cases 6) antibodies had little effect on the inactivation phase. The anti-C2 type 1, however, depressed inactivation by 50~60% of the control, suggesting that FVIII:C activated by APCC, persisted in the presence of anti-C2 type 1 antibodies.

Since anti-C2 mAbs (ESH4 and ESH8) represent the epitopes of anti-C2 type 1 and type 2 antibodies, respectively, APCC-mediated FVIII reactions were examined using specific anti-C2 mAbs (2 BU/ml) (Fig. 5B). ESH8 had little effect on APCC-mediated reactions, whilst ESH4 enhanced activation and moderated the subsequent inactivation. Kinetic analysis of these data showed that ESH4 inhibited inactivation by ~20% of the rate observed with control. The increased peak level of FVIII:C in the presence of ESH4 (~1.6-fold of control) appeared likely due to the relative enhancement of FVIII:C as a result of the significant delay in inactivation. An alternative anti-A2 mAb413 did not affect this reaction. These findings were consistent with the results obtained with polyclonal anti-FVIII antibodies.

APCC-mediated cleavage at Arg^{372} and Arg^{336} with anti-FVIII antibodies - To further confirm the effects of anti-FVIII antibodies on APCC-mediated activation, APCC-mediated cleavage of HCh was repeatedly examined. Representative time-course reactions for anti-FVIII antibodies with distinct properties and band densities of $A1^{1-372}$ are shown in Figs. 6A,B. When APCC without TF was added to FVIII, the $A1^{1-372}$ appeared within 5 min, followed by modest disappearance of this fragment. Cleavage at Arg^{372} using antibody from case 6 (anti-C2 type 2) was slightly delayed compared to those from case 1 (anti-A2 type 1) and case 3 (anti-C2 type 1). When APCC with TF was added to FVIII, the $A1^{1-372}$ appeared rapidly within 1 min, irrespective of the properties of antibodies. The $A1^{1-372}$ disappeared more slowly for case 3 antibody, however, compared to those from cases 1 and 6. This delayed inactivation of FVIII with anti-C2 type 1 antibody was attributed to significant inhibition of cleavage at Arg^{336}. Other anti-FVIII antibodies with same properties showed similar results (data now shown). Overall, the findings demonstrated that APCC-mediated FVIII activation was not

impaired by the presence of anti-FVIII antibodies, and that anti-C2 type 1 antibodies delayed the inactivation phase of APCC-mediated reaction, resulting in relatively persistent elevated levels of FVIII:C.

DISCUSSION

In this study, we have demonstrated that APCC could directly activate FVIII through limited proteolysis at Arg^{740} and Arg^{372}, followed by inactivation through proteolysis at Arg^{336}. This validity was confirmed by similar results obtained by different lots of APCC (data not shown). It seemed highly likely that these mechanisms contributed to the enhanced hemostatic effects of the combined APCC-FVIII reagents. Our initial hypothesis was that the enhanced effects were related to FVIII activation through the positive-feedback process due to generated thrombin induced by APCC, resulting in amplification of thrombin activity. The presence of FVIII and APCC, compared to APCC alone in a purified system, however, resulted not only in increased thrombin generation, but also in elevated FXa generation. The specificity of FXa generation assays was confirmed by including hirudin in the reaction mixtures. In addition, functional one-stage coagulation assays and SDS-PAGE analysis indicated that FVIIa/TF significantly participated as an initiator of the early phases of the APCC-mediated FVIII activation. The effects of PL in these mixtures appeared to be especially significant. Neither activation nor cleavage of FVIII by APCC occurred in the absence of PL, but was clearly demonstrated in its presence. Rapid reactions were more evident with both PL and TF. Surprisingly, thrombin derived from APCC did not appear to initiate FVIII activation in these circumstances. Taken together, rapid activation of FVIII by FVIIa/TF indicated that this complex more likely played a central role in the early APCC-mediated FVIII mechanisms, and FXa and thrombin appeared to participate in these reactions sequentially at later time points.

APCC-mediated FVIII activation and inactivation was dose-dependent, and even at low concentrations (0.05 U/ml) APCC had significant effects. It may be, therefore, that the FVIIa contained in standard doses of APCC (1 U/ml) administered for clinical purposes, might activate FX (and FIX) directly and predominantly rather than FVIII in circulating plasma. Consequently, the complete extrinsic FXase complex could be formed, and larger amounts of FXa and thrombin derived from APCC in this manner would promote thrombin generation and lead to fibrin formation. In the presence of small amounts of APCC with FVIII, however, the mechanisms of enhanced hemostasis described in this report could be pivotal. Further clinical investigations appear to be warranted to substantiate this novel mechanism *in vivo*.

A cell-based model, centered on FVIIa/TF is proposed as the principal initial mechanism of blood coagulation [20]. In this context, we have previously demonstrated that FVIII activation by FVIIa (1 nM) with TF was not abrogated by VWF *in vitro* [14], but it remained unclear whether this observation was significant physiologically. Our current findings on APCC-mediated FVIII reactions could help to clarify some of these multiple interacting mechanisms of blood coagulation *in vivo*. Our data supports the possibility that FVIIa/TF physiologically activates not only FX (and FIX), but also FVIII. Furthermore, FXa generated by FVIIa/TF in this manner could activate not only prothrombin, but also FVIII on PL surfaces in cell-based interactions.

APCC activated FVIII to a similar extent, irrespective of anti-FVIII antibodies with A2 or C2 epitopes, but it was evident that anti-C2 type 1 moderated the inactivation phase of the APCC-mediated reaction in association with the delayed cleavage at Arg^{336}. There were differences between anti-C2 type 1 and type 2 antibodies in the APCC-mediated reactions. APCC-mediated FVIII reactions depend on the activity of FVIIa/TF (and FXa) on PL surfaces, and it seemed possible that the effects of anti-C2 type 1 might be due to the inhibition of FVIII-PL interaction. The APCC-mediated effects on FVIII were minimal without PL, however, and there was no significant inhibition of FVIII-PL interaction in ELISA at concentrations used in the coagulation assay (data not shown). It seemed unlikely, therefore, that APCC-mediated mechanisms were related to inhibition of FVIII-PL interaction, although FVIII-PL interaction was inhibited at higher antibody-concentrations in ELISA (data not shown). Moreover, anti-C2 type 1 had little affect FVIII activation related to cleavage at Arg^{372} and Arg^{740}. We suggest, therefore, that these antibodies did not directly affect the APCC-mediated FVIII activation under the current conditions.

Our findings could provide challenging possibilities for the use of bypassing therapy in congenital and acquired HA patients with inhibitors, by administrating mixtures of FVIII and APCC, although this concept might be face that infused FVIII is rapidly cleared by inhibitors complexing to FVIII. Small amounts of APCC relative to the standard clinical dose (1 U/ml) could facilitate FVIII activation by FVIIa/TF on PL surfaces, even in patients with inhibitors. In addition, the enhanced hemostatic potential of APCC-FVIII could be beneficial for the control of symptomatic bleeding during immune tolerance induction therapy for HA with inhibitors. The specific characteristics of anti-FVIII antibodies appear to be critical in this respect, however, and it could be critical for therapeutic purposes to determine the nature of inhibitors in order to predict the effectiveness of combination therapy. Further studies appear to be warranted to assess APCC-mediated FVIII reactions *in vivo* in HA patients, especially those with anti-C2 type 1 antibody.

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FIGURE LEGENDS

Fig. 1. APCC-mediated activation of FVIII - (A) FVIII (10 nM) and PL (20 μM) were incubated with various concentrations of APCC and TF (0.5 nM). **(B)** FVIII (10 nM) and PL (20 μM) were incubated with APCC (0.05 U/ml) and various concentrations of TF. Samples in (A) and (B) were removed from mixtures at the indicated times, and the reactions were immediately terminated as described in Methods. FVIII:C was measured in one-stage clotting assays. FVIII:C prior to the addition of APCC represents the initial level. Experiments were performed at least four separate times and mean±S.D. are shown.

Fig. 2. The effect of FVIII on thrombin generated from APCC itself - PL (20 μM) and TF (0.5 nM) were mixed with S-2238 substrate in the absence or presence of FVIII (10 nM). Thrombin generation was initiated by the addition of APCC (0.05 U/ml), followed by measuring generated thrombin. The right hand vertical axis defines the ratios of the amounts of thrombin obtained in the presence of FVIII relative to those in its absence. The data obtained in assays without TF are also illustrated. Experiments were performed at least four separate times and mean±S.D. are shown.

Fig. 3. APCC-mediated proteolytic cleavage of FVIII - (A) FVIII (10 nM) in the absence **(a)** or presence **(b)** of hirudin (2.5 U/dl) was incubated with APCC (0.05 U/ml) for the indicated times in the absence or presence of PL (20 μM) and/or TF (0.5 nM). Aliquots were run on 8% gels followed by Western blotting using anti-A2 mAb. The right lane (VIIIa) illustrates control, thrombin-cleaved

FVIII. **(B)** In similar experiments, FVIII (10 nM) and PL (20 μM) were incubated with APCC (0.05 U/ml) in the absence or presence of TF (0.5 nM) or E-76 (2.5 μM) for the indicated times. Aliquots were run on 8% gels followed by Western blotting using anti-A1 mAb **(a)**. Data obtained by quantitative densitometry of the $A1^{1-372}$ in (B) are shown in **(b)**. Band densities of $A1^{1-372}$ were expressed as the ratio relative to those in $A1^{1-372}$ in FVIIIa (control).

Fig. 4. The role of FVIIa in the generation of FXa from APCC solution with FVIII - FVIII (10 nM) and PL (20 μM) were incubated with S-2222 substrate in the absence or presence of E-76 (2.5 μM) and TF (0.5 nM). FXa generation was initiated by the addition of APCC (0.05 U/ml). The reaction was terminated at the indicated times, and the amounts of generated FXa were measured. To completely exclude FVIII activation by generated thrombin, hirudin (2.5 IU/ml) was added to the samples prior to the addition of APCC. Experiments were performed at least four separate times and mean±S.D. are shown.

Fig. 5. APCC/TF-mediated FVIII activation with anti-FVIII inhibitors - FVIII (10 nM) was mixed with anti-FVIII antibody-$F(ab')_2$ (2 BU/ml) or control-$F(ab')_2$ (500 nM) for 2 hr prior to incubation with APCC (0.05 U/ml), TF (0.5 nM), and PL (20 μM). The results for cases 1-6 from HA patients and those for three mAbs are shown in **(A-a** and **B)**, respectively. Samples in (A) and (B) were taken from the mixtures at the indicated times, followed by terminating the reactions. FVIII:C was measured in one-stage clotting assays. FVIII:C prior to the addition of APCC represents the initial level. **(A-b)** From the data in (A-a), the ratios of peak activities of FVIII activation with antibody-$F(ab')_2$ relative to control are drawn in *closed bars*. The peak FVIII:C levels after the addition of APCC were regarded as 100%. Inactivation rates were calculated using the formula of single exponential decay. The ratios of apparent inactivation rate of FVIII(a) with antibodies relative to control are drawn in *open bars*.

Fig. 6. APCC-mediated cleavage of A1 with anti-FVIII inhibitors - (**A**) FVIII (10 nM) was preincubated with antibody-$F(ab')_2$ (2 BU/ml) from case 1 (**a**), case 3 (**b**), and case 6 (**c**) for 2 hr prior to incubation with APCC (0.05 U/ml), TF (0.5 nM), and PL (20 μM). Samples were run on 8% gels followed by Western blotting using anti-A1 mAb. Data obtained by quantitative densitometry of $A1^{1-372}$ in (A) are shown in (**B**). Band densities of $A1^{1-372}$ were expressed as the ratio relative to $A1^{1-372}$ in FVIIIa (control).

Table 1. Properties of anti-FVIII inhibitor antibodies

Case	Type	Epitope	Type of kinetics	Inhibitor titer (BU/mg)
1	alloAb	A2	1	113
2	alloAb	A2	1	57
3	alloAb	C2	1	587
4	alloAb	C2	1	127
5	autoAb	C2	1	20
6	alloAb	C2	2	4
413	mAb	A2	1	59,500
ESH4	mAb	C2	1	39
ESH8	mAb	C2	2	10,000

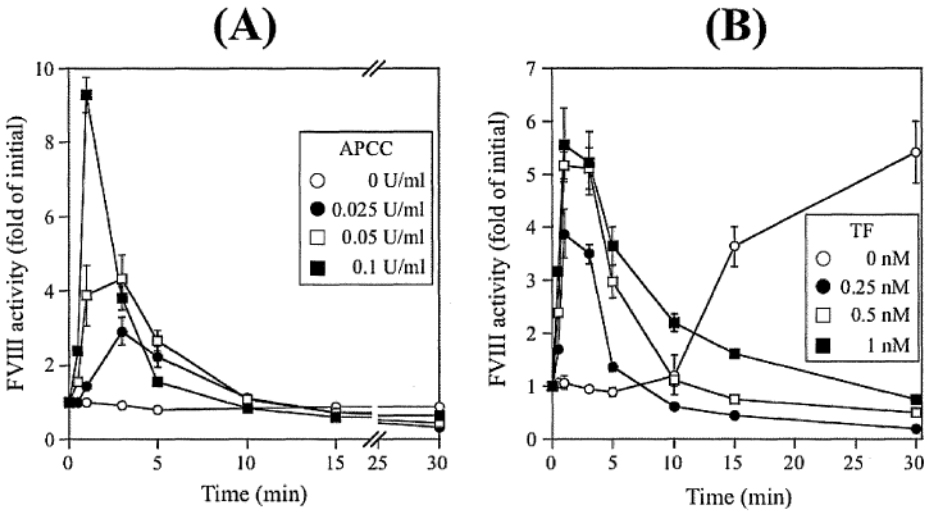

Figure 1

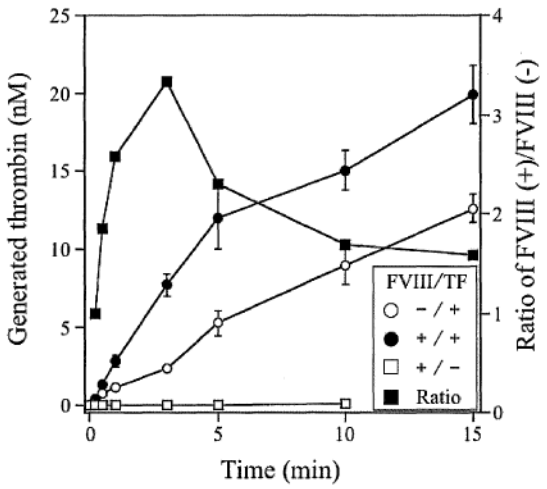

Figure 2

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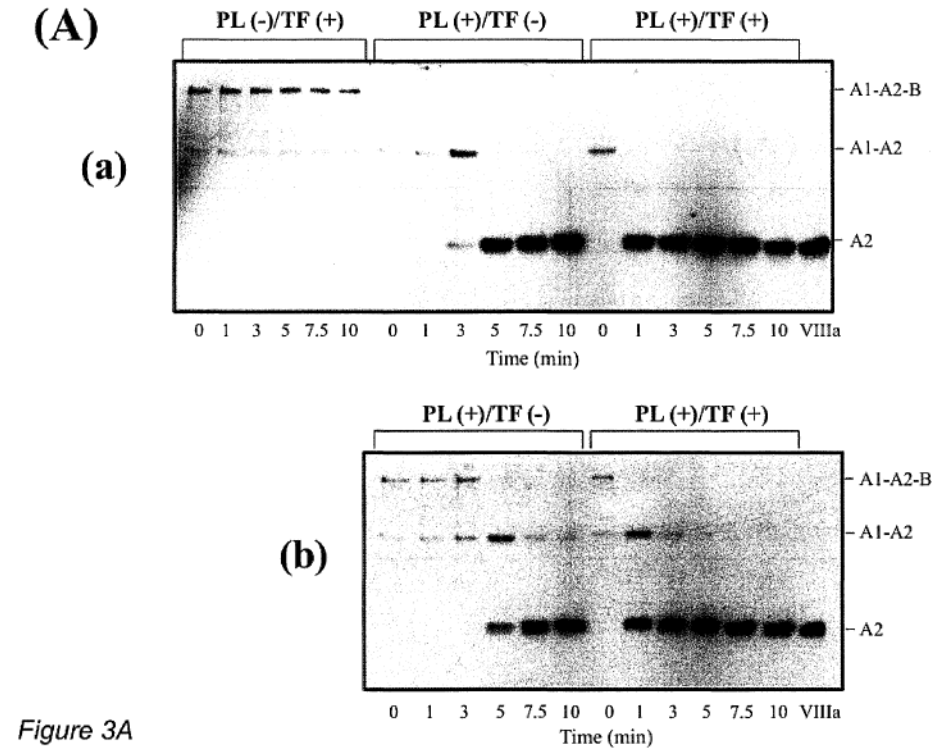

Figure 3A

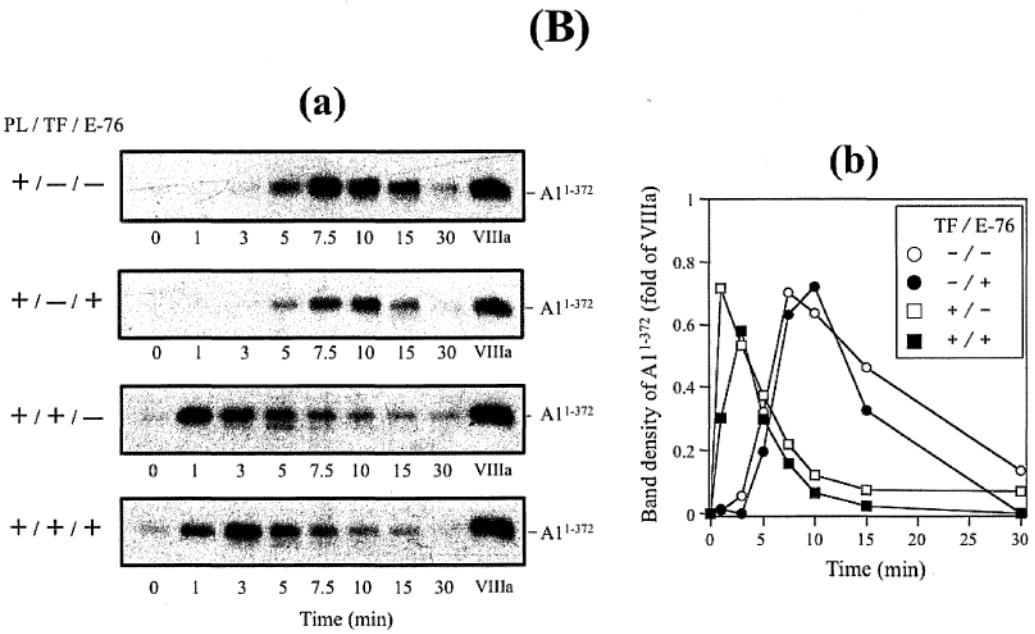

Figure 3B

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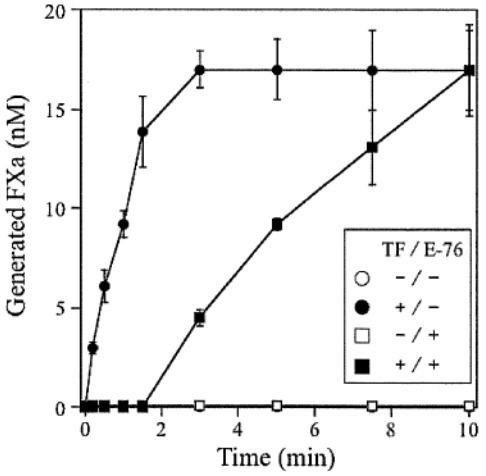

Figure 4

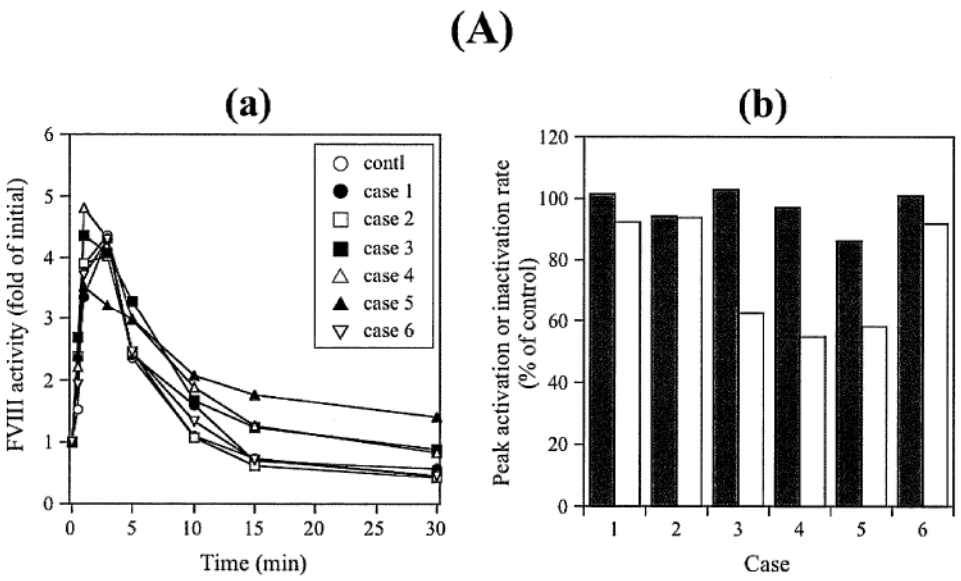

Figure 5A

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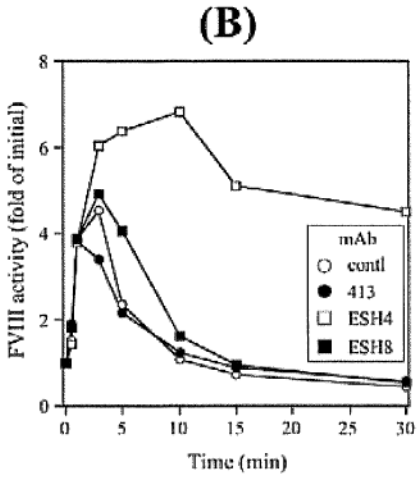

Figure 5B

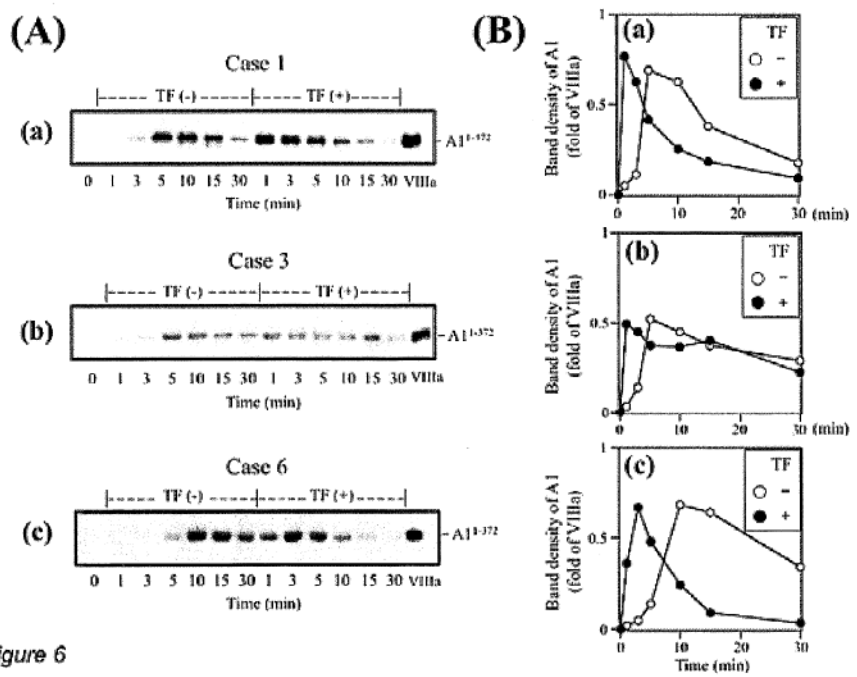

Figure 6

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