Emicizumab, the bispecific antibody to factors IX/IXa and X/Xa, potentiates coagulation function in factor XI-deficient plasma in vitro

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Essentials

- Emicizumab mimics factor (F)VIIIa cofactor function, augments the intrinsic tenase activity.
- We assessed the emicizumab-driven hemostatic function in FXI-deficient plasmas.
- Emicizumab improved the coagulation potentials in severe FXI-deficient plasma.
- Emicizumab may provide a possibility for clinical application in patients with FXI deficiency.

Summary. *Background:* Patients with factor (F)XI deficiency commonly present with markedly prolonged activated partial thromboplastin times (APTT), although bleeding phenotypes are heterogeneous. Emicizumab, a bispecific monoclonal antibody to FIX/FIXa and FX/FXa, mimics FVIIIa cofactor function on phospholipid (PL) surfaces. Antibody reactions were designed, therefore, to augment mechanisms during the propagation phase of blood coagulation. *Aim:* To assess emicizumab-driven hemostatic function in FXI-deficient plasmas. *Methods and Results:* Standard ellagic acid (Elg)/PL-based APTTs of different FXI-deficient plasmas (n = 13; FXI activity, < 1 IU dl^{-1}) were markedly shortened dose dependently by the presence of emicizumab. To further analyze the effects of emicizumab, clot waveform analysis (CWA) in FXI-deficient plasmas with emicizumab, triggered by tissue factor (TF)/Elg demonstrated improvements in both clot times, reflecting the initiation phase, and coagulation velocity, which represents the propagation phase. Emicizumab also enhanced the TF/Elg-triggered thrombin generation in FXI-deficient plasmas dose-dependently although the degree of enhancement varied in individual cases. Thrombin generation with either FVII-deficient plasma or FIX-deficient plasma treated with anti-FXI antibody showed little or no increase by the co-presence of emicizumab, suggesting that the accelerated thrombin generation in FXI-deficient plasmas by emicizumab should depend on the FIXa-involved coagulation propagation initially triggered by FVIIa/TF. The ex vivo addition of emicizumab to whole blood from three patients with severe FXI deficiency demonstrated modest, dose-dependent improvements in Ca^{2+}-triggered thromboelastograms (NATEM mode). *Conclusion:* Emicizumab appeared to improve

coagulation function in severe FXI-deficient plasma, and might provide possibilities for clinical application in patients with FXI deficiency.

Keywords: clot waveform analysis; emicizumab; factor XI deficiency; plasma; thrombin generation assay.

Introduction

Factor (F)XI is the zymogen of a serine protease, activated FXI (FXIa), which contributes to the blood coagulation process by converting FIX to FIXa [1]. FXI(a) is a 160-kDa homo-dimer [2,3] that circulates in blood at a concentration of 20–30 n M in complex with high-molecular-weight kininogen (HMWK) [4]. FXIa-mediated FIX activation accelerates amplification and propagation of thrombin generation that is initiated by the FVIIa and tissue factor (TF) complex. Conversion of FIX to FIXa requires the proteolytic cleavages of scissile bonds between Arg145-Arg146 and Arg180-Arg181, resulting in the release of an activation peptide [5]. These Ca^{2+} -dependent reactions are catalyzed by the FVIIa/TF on phosphatidylserine-rich phospholipid (PL) micelles [6] and by FXIa in a PL-independent process [1,5,7]. Considerable interest has focused recently on FXIa as a therapeutic target in thrombosis [8,9]. FXI deficiency is inherited as an autosomal trait (historically also named hemophilia C), with the FXI gene located on chromosome 4q35 [10]. The deficiency is commonly detected in Ashkenazi Jews and occurs at a frequency of approximately 10% in this population [11]. Based on FXI coagulation activity (FXI:C), the genotypes are classified as a homozygote or heterozygote [3]. In general, homozygotes present with a FXI:C of less than 15 IU dl^{-1} together with a marked prolongation of the activated partial thromboplastin time (APTT). Heterozygotes present with a FXI:C of 15–70 IU dl^{-1} and a modestly prolonged or normal APTT [11,12]. Patients with FXI deficiency frequently suffer from delayed bleeding after injury or surgery, easy bruising and menorrhagia, but these clinical symptoms are less severe than severe hemophilia A or B, and are heterogeneous irrespective of the FXI:C levels [13–15]. FXI replacement therapy is available for patients with significant hemorrhage or requiring hemostatic management for major surgery, usually in the standard form of fresh-frozen plasma or plasma-derived and recombinant FXI concentrates [16]. Emicizumab (also termed ACE910) is a humanized, recombinant bispecific antibody to FIX/FIXa and FX/FXa that mimics the cofactor function of FVIIIa by positioning FIXa and FX to promote the enzymatic activity of FIXa in the tenase complex [17,18]. In the phase 1/2 study and phase 3 studies in patients with severe hemophilia A, once-weekly subcutaneous administration of emicizumab was shown to be well tolerated and substantially effective in preventing bleeding episodes irrespective of the presence of inhibitors [19–21]. The effective therapeutic range of emicizumab (10–100 μg ml^{-1} in plasma) in the phase 1/2 study potentiated by up to a maximum of ~ 20% thrombin generation compared with that mediated by the native-tenase complex [19,22]. Therefore, we hypothesized that emicizumab may compensate for the lack of FXI-mediated coagulation propagation in the FXI-deficient state by enhancing a tenase potential mediated by FIXa produced from FVIIa/TF. In this context, we have examined the in vitro effects of emicizumab on the hemostatic function using FXI-deficient plasmas, and have investigated the potential

clinical use of emicizumab for therapy in patients with FXI deficiency.

Materials and methods

Reagents

A recombinant emicizumab, a humanized bispecific IgG4 antibody to FIX/FIXa and FX/FXa, was produced from a Chinese hamster ovary (CHO) cell line [17,18]. A recombinant FIX (Benefix; Pfizer, New York, NY, USA) and FVIIa (Novoseven; NovoNordisk, Bagsværd, Denmark) were purchased from the indicated vendors. Human FX and FXa (Hematologic Technologies, Inc., Burlington, VT, USA), FVIIa-specific inhibitor peptide E-76 (Ac-ALCDDPRVDRWYCQFVEG-NH□; Bachem, Bubendorf, Switzerland) and FXa substrate S-2222 (Chromogenix, Diapharma Inc., West Chester, OH, USA) were purchased from the indicated vendors. An anti-FXI monoclonal antibody (mAb; XI-5108) was prepared from the hybridoma and a property on specific reactivity and high affinity for FXI/FXIa was previously reported [23]. A specific for calcium-dependent conformation of mAb3A6 against the heavy chain of FIX was prepared [24]. An anti-human TF neutralizing antibody was generated from immunized mice and then we humanized it to human IgG4κ. The humanized anti-TF antibody was also expressed in CHO cells by a recombinant method and purified. Plasmas from FXI-deficient patients (n = 10, different batches), FVII-deficient patients and FIX-deficient patients were commercially purchased from George King Bio-Medical (Overland Park, KS, USA). The APTT reagent containing ellagic acid (Elg) and PL (Thrombocheck APTT-SLA; Sysmex, Kobe, Japan), silica-based APTT-HemosIL (Instrumentation Laboratory, Bedford, MD, USA), recombinant human TF (Innovin®; Dade, Marburg, Germany), ellagic acid (Elg; Sysmex, Kobe,

Japan) and the thrombin-specific fluorogenic substrate (Z-Gly-Gly-Arg-AMC; Bachem, Bubendorf, Switzerland) were purchased from the indicated vendors. PL vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine and 30% phosphatidylethanolamine were prepared as previously reported [25].

Plasma samples

The study was approved by the Medical Research Ethics Committees of Nara Medical University and Chugai Pharmaceutical Co., Ltd, and blood samples were obtained after informed consent following local ethical guidelines. Fresh FXI-deficient plasmas were obtained from three patients with severe FXI deficiency. Normal pooled plasma was prepared from 20 healthy volunteers. Whole blood samples were obtained by venipuncture into plastic tubes containing one-tenth of volume of 3.2% (w/v) trisodium citrate. Samples were centrifuged for 15 min at 1500 ×g, stored at -80 °C, and thawed at 37 °C immediately prior to the assays.

Clotting factor activity assays

Procoagulant activities of various clotting factors in FXI-deficient plasmas were measured in a one-stage clotting assay on the STart4 automated instrument (Stago, Gennevilliers, France). Mean and standard

deviation of FII, FVII, FVIII, FIX, FX and FXI:C levels in all of the FXI-deficient samples (n = 13; 10 commercial samples, three our patients' samples) were 106±9, 115±10, 116±15, 112±18, 89±8 and < 1.0 U dl^{-1}, respectively, again confirming an isolated FXI deficiency.

Preparation of plasma samples with emicizumab

FXI-deficient plasmas were incubated with the indicated concentrations of emicizumab for 1 h at 37 ° C. FVII-deficient plasma and FIX-deficient plasma were preincubated with anti-FXI mAb (100 μg ml^{-1}; approximately 660 nM) for 1 h at 37 ° C, followed by a further 1-h incubation
with emicizumab at 37 °C. A shorter time reaction with emicizumab and either FXI-, FVII- or FIX-deficient plasma also demonstrated the similar results to that at 1 h-reaction (data not shown).

Clot waveform analysis (CWA)

Clot waveform analysis was performed on the CS2000i (Sysmex, Kobe, Japan) using two different trigger reagents, based on Elg and PL (APTT reagent), and Elg (final concentration, 0.3 μM) mixed with TF (0.1 pM) and PL (10 μM) (mixed reagent) [26]. This mixed reagent was previously defined for hemostatic monitoring of bypassing therapy in hemophilia A patients with inhibitor [27]. Clot formation was initiated by the addition of $CaCl_2$ (20 mM). Light transmittance changes during the coagulation process were monitored as the clot waveform pattern, and traces were computer-processed using the commercial kinetic algorithm. The first derivative of the transmittance reflects the coagulation velocity. The clot time (CT), defined as the time until the start of coagulation, and maximum coagulation velocity (|min1|) were recorded.

Thrombin generation assay (TGA)

Thrombin generation was assayed using TF (1 pM) and ellagic acid (Elg, 0.3 μM) with PL vesicles (4 μM), and modified to be sensitive to intrinsic activity as well as extrinsic activity as previously described [27]. Briefly, 80 μl test plasma was mixed with 20 μl trigger reagent (TF/Elg/PL) in microtiter wells (ThermoLab System, Helsinki, Finland) and the assay initiated by the addition of 20 μl of 100 mM $CaCl_2$ together with 5 mM fluorogenic substrate. The development of fluorescent signals was monitored at 8-s intervals using a Fluoroskan Ascent microplate reader (Thermo Electron Co., Waltham, MA, USA) with 390 nm (excitation) and 460 nm (emission) filter set. Thrombin generation (nM) was calculated by reference to standard thrombin calibrator samples. Data analyses were performed using Thrombinoscope software (Maastricht, The Netherlands), and the parameters, peak thrombin and endogenous thrombin potentials (ETP) were recorded.

Rotational thromboelastometry (ROTEM)

Rotational thromboelastometry was performed 30 min after venipuncture using the Whole Blood Hemostasis Analyzer® (Pentapharm, Munich, Germany). The viscosity of clot formation was monitored in whole blood samples (300 μl) obtained from three of our patients with FXI deficiency mixed with 20

μl $CaCl_2$ (100 mM). The coagulation process was quantified using the clotting time (CT; the time from the start of measurement until detection of clot firmness at 2-mm amplitude) and the clot formation time (CFT; the time from the initiation of clotting until detection of clot firmness at 20-mm amplitude).

FVIIa/TF-catalyzed FIX cleavage

Recombinant FIX (500 nM) preincubated with emicizumab (50 μg ml^{-1}) was mixed with FVIIa (10 nM)/TF (1 nM) in the presence of PL (20 μM) in hepes-buffered saline buffer containing 5 mM $CaCl_2$ at 37 °C. Aliquots were removed at the indicated times and the reactions were immediately terminated and prepared for SDS-PAGE by adding SDS and boiling for 3 min.

Electrophoresis and western blotting

SDS-PAGE was performed using 8% gels at 150 V for 1 h, followed by western blotting analysis. Protein bands were probed using anti-FIX mAb3A6, followed by goat anti-mouse peroxidase-linked secondary mAb. Signals were detected using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA).

FVIIa/FIX-catalyzed FXa generation assay

Various concentrations of FIX were reacted with FVIIa/TF (1 nM/0.1 nM) with PL vesicles (20 μM). FVIIa activity was terminated after 1 min by the addition of an inhibitor peptide of FVIIa, E-76 (2.5 U ml^{-1}), and FXa generation was initiated by the addition of FX (200 nM). Emicizumab (100 μg ml^{-1}) was added prior to addition of FVIIa/TF or prior to addition of FX. Aliquots were removed at appropriate times to assess the initial rates of product formation and were added to EDTA-containing tubes to quench the reactions. Rates of FXa generation were determined by the addition of chromogenic substrate specific to FXa, S-2222 (0.46 mM). Reactions were read at 405 nm using a Labsystems Multiskan Multisoft microplate reader (Labsystems, Helsinki, Finland). Control experiments demonstrated that the FX preparation used in this study was not contaminated with FXa (data not shown).

Data analysis

Experiments in all samples were performed at least three separate times, and data are shown as the average and standard deviation (SD) or standard error of mean (SEM).

Results

APTTs of FXI-deficient plasmas in the presence of emicizumab

The effects of emicizumab on coagulation function in FXI-deficient plasma were initially assessed using 10 commercial samples and plasma from our three patients. Standard APTTs were recorded after the addition of activating reagent containing Elg, PL and $CaCl_2$. The APTTs of FXI-deficient plasmas in the

absence of emicizumab were markedly prolonged (> 90 s) and were markedly shortened dose dependently in the presence of the antibody. Emicizumab concentrations up to 100 μg ml^{-1} (corresponding to the plasma concentration in the high-dose clinical cohort of hemophilia A patients [19,20])
shortened the APTTs to approximately 40 s with saturable levels (Fig. 1). Similarly, the addition of emicizumab to normal plasma progressively shortened the APTTs from 34.5 to 21.5 s with saturable levels. The degree of change at the maximum concentration of emicizumab appeared to be greater in the FXI-deficient plasmas (57%) than in the normal plasma (38%) (Fig. 1 *inset*). In addition, the similar experiment by a silica-based APTT assay, instead of Elg, was repeated, and the obtained result was similar to that by an Elg-based APTT assay (data not shown).

TF/Elg trigger CWA in FXI-deficient plasmas with emicizumab

The mode of action of emicizumab mimics FVIIIa cofactor activity, and earlier reports have indicated that assessment of intrinsic coagulation using APTT-based assays might not provide a reliable estimate of antibody responses [17,19 – 21]. In this context, we have previously described the use of a mixed activation reagent, TF/Elg/PL, reflecting both intrinsic and extrinsic pathways, to study emicizumab-driven coagulation activity [28]. We extended our present investigations, therefore, to examine emicizumab-related mechanisms in FXI-deficient plasma in comparison with normal plasmas using TF/Elg-triggered CWA as described in Methods. Figure 2(A) illustrates representative clot waveforms in FXI-deficient plasmas using emicizumab at different concentrations. The various parameters derived from CWA are illustrated in Fig. 2(B, C). The clot time (CT), reflecting the initiation phase in the coagulation process, was shortened dose dependently in both FXI-deficient plasma (by 10% at 100 μg ml^{-1}) and normal plasma (by 17%) (Fig. 2B and *inset*). The coagulation velocity (|min1|), reflecting the propagation phase, demonstrated the improved effects of emicizumab in both FXI-deficient plasma (by ~ 1.2-fold) and normal plasma (by ~ 1.6-fold) (Fig. 2C and *inset*). The results suggested that emicizumab had a meaningful impact on the coagulation propagation pathway independently of a pivotal role of FXI(a). Similar experiments on the Elg-triggered CWA were repeated, and the significant improvement of parameters, in particular |min1|, in the emicizumab-reacted FXI-deficient plasmas was observed (Data S1), whereas those on TF-triggered CWA one any little showed the improvement effects (data not shown).

Impact of emicizumab on thrombin generation in FXI-deficient plasmas

The potentiating nature of emicizumab observed in the global assays of coagulation described above was studied in more detail using TF/Elg-triggered thrombin generation assays as defined in Methods. Emicizumab, dose dependently, mildly enhanced thrombin generation potential in FXI-deficient plasmas (Fig. 3A). The peak thrombin measurements (Fig. 3B) and the ETP calculations (Fig. 3C) demonstrated dose-dependent increases up to approximately 100 μg ml^{-1} emicizumab. These measurements, however, appeared to vary in individual samples, resulting in a wide range of standard error. Peak levels of thrombin generation observed in FXI-deficient plasmas in the presence of emicizumab (10 – 100 μg ml^{-1}) were close

to those obtained without emicizumab in the representative plasma supplemented with purified plasma-derived FXI at 1.0 – 1.5 n M (corresponding to approximately 5 IU dl^{-1} of FXI:C; Fig. 3D). On the other hand, a similar approach by Elg-trigger failed to detect thrombin generation, and the TF-trigger approach showed that the significant dose-dependent enhancing effect appeared unlikely to be observed (data not shown).

Mechanism of enhanced thrombin generation in FXI-deficient plasma with emicizumab

Earlier studies have established that emicizumab is dependent on FIXa for tenase activation on PL surfaces [17,28], and our present results suggested that FVIIa/TF-mediated FIXa generation in both FXI-deficient plasmas and normal plasmas contributed to the effectiveness of the antibody. Therefore, experiments on the mode of action of emicizumab were further developed in circumstances in the absence of FXI using FVII-deficient plasma and FIX-deficient plasma as described in Methods. FVII-deficient plasma was preincubated with anti-FXI mAb to neutralize FXI:C, followed by a further incubation with various amounts of emicizumab prior to measurement of thrombin generation triggered by Elg/TF. Normal plasma incubated with anti-FXI mAb was regarded as a positive control (FXI-depleted plasma). A representative thrombogram is illustrated in Fig. 4(A). In the absence of emicizumab, very little thrombin generation in FVII-deficient FXI-depleted plasma was observed relative to FXI-depleted one, and addition of emicizumab did not increase thrombin production.

In similar experiments, FIX-deficient plasma was preincubated with anti-FXI mAb prior to measurements of thrombin generation. A representative thrombogram is illustrated in Fig. 4(B). In the absence of emicizumab, peak thrombin levels were low, and lag time and time to peak values were modestly delayed in FIX-deficient FXI-depleted plasma. Also, in the presence of emicizumab little change in thrombin generation was evident (Fig. 4B). Overall, therefore, the findings were in keeping with the pathway of FVIIa/TF-mediated FIXa generation in FXI-deficient plasma contributing to the potential for emicizumab to enhance FXa generation following thrombin generation.

Emicizumab does not affect the FVIIa–FIX association

To investigate the influence of emicizumab on the association of FVIIa and FIX in FXI-deficient plasma, the effect of emicizumab on FIX cleavage by FVIIa/TF was examined. FIX (500 nM) in the absence or presence of emicizumab (50 μg ml^{-1}) was reacted with FVIIa (10 nM)/TF (1 nM) and PL (20 μM), prior to SDS-PAGE/western blotting analysis. Proteolytic cleavage of the HCh at Arg145 and Arg180 in FIX was visualized using an anti-FIX HCh mAb3A6 (Fig. 5A). By the addition of FVIIa/TF, FIX was initially rapidly converted to FIX a (cleavage at Arg145 [5]), followed by the time-dependent production of FIXa (cleavage at Arg180 [5]). This cleavage pattern was similar in the absence (*upper panel*) and presence (*lower panel*) of emicizumab, and rather slightly slowly in its presence. At t = 0 in the reactant mixture of FIX and emicizumab, two additional bands (shows the arrows) were visualized, and the densities of both

bands did not change time dependently. These bands were derived from the binding of secondary mAb to the heavy and light chains of emicizumab (data not shown).

An effect of emicizumab on FVIIa/FIX-catalyzed FXa generation was further investigated. Various concentrations of FIX and PL (20 μM) were incubated with FVIIa/TF (1 nM/0.1 nM). FVIIa activity was terminated after 1 min by the addition of E-76, and FXa generation was initiated by the addition of FX (200 nM) as described in Methods. Emicizumab (100 μg ml^{-1}) was added prior to reaction with FVIIa/TF or prior to addition of FX (Fig. 5B). Under both circumstances, with addition of emicizumab, FXa generation showed similar levels. Taken together, these results demonstrated that emicizumab did not affect the FVIIa/TF – FIX association.

Ca^{2+} ROTEM in FXI-deficient whole blood with emicizumab

The *ex vivo* effects of emicizumab on global hemostasis in whole blood obtained from three patients with FXI deficiency were studied using the Ca^{2+}-triggered ROTEM (NATEM) technique as described in Methods. A representative thromboelastogram for each case is illustrated in Fig. 6. The calculated ROTEM parameters, CT and CT + CFT, are shown in each figure. The median 95% confidence intervals (CIs) of CT and CT + CFT obtained in 20 normal controls were 711 – 1025 and 929 – 1528 s, respectively. Thromboelastogram traces demonstrated little evidence of coagulation in native FXI-deficient whole blood within 60 min for two patients (Fig. 6A and B) but somewhat reduced coagulation potential relative to control in native FXI-deficient whole blood for one patient (Fig. 6C), supportive of heterogeneity by NATEM of coagulation potential in patients with FXI deficiency [29]. The *ex vivo* addition of emicizumab, however, showed modest dose-dependent improvements in both CT and CT + CFT for all of the patients, although these parameters remained lower than those in normal whole blood. In case (A), the hemostatic effect of emicizumab was seen at low concentration of 10 μg ml^{-1}, and appeared to be maximal at 60 μg ml^{-1} and be mildly diminished at 120 μg ml^{-1}. In case (B), the hemostatic effect of emicizumab was observed weakly at concentration of 30 μg ml^{-1}, and appeared to be slightly augmented at concentrations of both 120 and 240 μg ml^{-1}. In case (C), the hemostatic effect of emicizumab appeared unlikely to be enough at 10 μg ml^{-1}, but was mildly dose dependent at greater than 30 μg ml^{-1}. These results demonstrated that emicizumab might improve hemostatic potential in patients with FXI deficiency, although the improvement effects varied between patients.

Discussion

FXI is proteolytically activated by (i) FXIIa in the presence of prekallikrein (PK) and HMWK in the early contact reaction and by (ii) thrombin, bypassing these mechanisms and acting as a positive feedback [30]. The modern concept of physiological hemostasis is based on cell-based coagulation [31]. In this model, initial tissue injury leads to activation of FIX by the FVIIa/TF complex and mediates tenase activity, which generates thrombin. This reaction is rapidly terminated by tissue factor pathway inhibitor (TFPI) [32], but

sufficient FXIa is produced to maintain activation of FIX in the presence of Ca^{2+} [30] and consolidate the formation of tenase with FVIIIa as a cofactor in the propagation phase of coagulation. In the patients with severe FXI deficiency, therefore, tenase formation is markedly decreased and thrombin generation is significantly impaired. Emicizumab is a bispecific monoclonal antibody to FIX/FIXa and FX/FXa that mimics FVIIIa cofactor function on PL membranes [17,18], and could be expected to augment the FIXa-mediated coagulation propagation pathway initially triggered by TF/FVIIa in severe hereditary coagulation disorders, including FXI deficiency.

In our studies, emicizumab enhanced coagulation potential in FXI-deficient plasmas in vitro. Our conclusions were based on several original observations as follows. (i) Emicizumab shortened the clot time (CT) in Elg/TF-triggered CWA in FXI-deficient plasmas and an improvement in |min1| was observed. (ii) Emicizumab enhanced thrombin generation in Elg/TF-triggered TGA in FXI-deficient plasmas. (iii) In addition, thrombin generation in either FVII-deficient plasma or FIX-deficient plasma incubated with anti-FXI antibody was not affected by the presence of emicizumab. (iv) Emicizumab did not affect FVIIa/TF-induced FIX activation. (v) NATEM demonstrated the emicizumab-mediated enhanced coagulant effect mildly. All assays showed that the emicizumab-enhanced coagulation potential in FXI deficiency was mild compared with normal plasma including FVIIIa. The data could support the role of FIXa in emicizumab's potentiating coagulation propagation of the intrinsic tenase activity in FXI-deficient plasma, because FXIa-induced FIXa generation does not occur in FXI-deficient plasma. On the other hand, TF-triggered CWA and TF- or Elg-triggered thrombin generation assay did not show the enhancing effect of emicizumab. Although emicizumab could improve the coagulation potentials in FXI-deficient plasma, its observed effect depended on the trigger condition and measurement approach. Based on our findings, we have schematically demonstrated the putative coagulation-enhancing mechanism of emicizumab in the FXI-deficient condition (Fig. 7).

The shortening of APTT by emicizumab in FXI-deficient plasma greatly attracted our interest. We had considered that emicizumab might not accelerate FX activation so much in FXI-deficient plasma, because either TF or FXIa was not theoretically present in citrated plasma from patients and thus FIX activation might not be efficiently induced in the APTT-based assay. The anti-FXI antibody and anti-TF antibody were added to FXI-deficient plasma to preclude the possibility of trace amounts of FXI(a) and TF, but the APTT in FXI-deficient plasma with and without emicizumab was not affected (data not shown). Even by the trigger of PL alone without Elg (representing PTT), FXI-deficient plasma with and without emicizumab revealed the clot formation (Data S2). During the APTT reaction of the FXI-deficient plasma, therefore, the FIX activation did not occur but fibrin formation occurred through the downstream coagulation reaction. On the other hand, we found that addition of emicizumab shortened APTT dose dependently. As emicizumab's cofactor action requires FIX/FIXa [33], we think that a trace amount of FIXa should exist in FXI-deficient plasma. Because we have no effective way to measure the trace amounts of plasma FIXa,

the origin of FIXa in FXI-deficient plasma is unclear (e.g. the trace amounts of FIXa exist in circulating plasma or FIX is converted to FIXa as a result of a technical event during blood collection). The former hypothesis that FIXa exists in the circulating blood is interesting, but its physiological roles in thrombosis and hemostasis remain to be clarified. In the present study, although the origin of FIXa is unclear, even APTT-based FXI-deficient plasma could lead to amplification of emicizumab activity, and the subsequent experiments could be significantly developed. The effects of emicizumab in TGA in our investigations varied modestly with different FXI-deficient plasma samples. It is known that thrombin generation is affected by the activity and antigen of individual clotting factors, and appears to be inconsistent even at similar concentrations of FXI [34]. In addition, it may be that the degree of FIX activation by FVIIa/TF in our samples contributed to the disparate assays. Therefore, the difference in the enhancing effects of emicizumab in FXI-deficient plasma samples may be not surprising.

Hemostatic treatment in patients with severe FXI deficiency commonly depends on replacement therapy with fresh-frozen plasma and plasma-derived and recombinant FXI products [16]. These types of treatment are known, however, to be associated with risks of virus transmission, the consequences of fluid imbalance and the rare development of anti-FXI alloantibody inhibitors in multi-transfused homozygous patients [35]. Our results from in vitro and ex vivo experiments, including ROTEM using whole blood, pointed to the possibility of improved global coagulation mediated by emicizumab. The results varied in individual cases, but nevertheless, further investigations appear to be warranted to determine the potential clinical use of the antibody for prophylaxis in patients with FXI deficiency.

Addendum

H. Minami performed experiments, analyzed the data and prepared figures. K. Nogami designed all experiments, analyzed the data, interpreted the data, prepared figures, and wrote and edited the manuscript. K. Yada and K. Ogiwara performed experiments, analyzed the data and prepared figures. S. Furukawa performed experiments and interpreted the data. T. Soeda and T. Kitazawa prepared emicizumab and anti-FXI monoclonal antibody, XI-5108. M. Shima supervised the manuscript.

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Disclosure of Conflict of Interest

H. Minami, K. Ogiwara, K. Yada, S. Furukawa, K. Nogami and M. Shima receive research support from Chugai Pharmaceutical Co., Ltd (Chugai) and are engaged in clinical studies sponsored by Chugai and F. Hoffmann-La Roche. K. Nogami and M. Shima receive (consulting) honoraria from these companies and

are inventors of the patents relating to emicizumab. T. Soeda and T. Kitazawa are employees of Chugai, possess Chugai's stock and are inventors of patents relating to emicizumab. K. Nogimai further reports grants and personal fees from Baxalta Inc. and Novo Nordisk A/S, and grants from Biogen Inc., Bayer AG and Sysmex Co. outside the submitted work.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Elg-triggered CWA in the FXI-deficient plasmas with emicizumab. FXI-deficient plasmas were incubated with various concentrations of emicizumab, followed by measurement of clot waveform triggered by Elg as described in Methods. A representative dose-dependent clot waveform in one case is illustrated in (A). The obtained parameters, CT (B) and |min1| (C) were plotted as a function of emicizumab in the FXI-deficient plasma. The average values and SEM are shown. Some bars for SEM were shorter than the symbols.

Data S2. PL-triggered PTT in the FXI-deficient plasmas with emicizumab. FXI-deficient patients' plasmas (opened circles) or normal pooled plasma (closed circles) were incubated with various amounts of emicizumab, and clotting times were measured in the CS2000i instrument after the addition of the PTT reagent, PL (10 μM) and $CaCl_2$ (20 mM). Experiments with each sample were performed at least three separate times and the average values and SEM are shown.

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Figure Legends

Fig. 1. Elg/PL-triggered APTT in FXI-deficient plasmas with emicizumab: FXI-deficient patients' plasmas (opened circles) or normal pooled plasma (closed circles) were incubated with various amounts of emicizumab, and clotting times were measured in the CS2000i instrument after the addition of the PTT reagent (Elg/PL, 0.3 μM/10 μM) and $CaCl_2$ (20 mM). Insets: Clotting times in samples without emicizumab were regarded as the initial, and the results with emicizumab were expressed as the fold of the initial. Experiments with each sample were performed at least three separate times and the average values and SEM are shown. Some bars for SEM were shorter than the symbols. Elg, ellagic acid; APTT, activated partial thromboplastin time; SEM, standard error of mean.

Fig. 2. CWA in FXI-deficient plasma in the presence of emicizumab: (A) FXI-deficient plasmas (n = 13) were incubated with various amounts of emicizumab (0 – 100 μg ml^{-1}), followed by measurement of clot waveform triggered by Elg/TF as described in Methods. A representative dose-dependent clot waveform in one case is illustrated in (A). The obtained parameters, CT (B) and |min1| (C) were plotted as a function of emicizumab in the FXI-deficient plasma. The *insets* in (B) and (C) show the CT and |min1| obtained in CWA on the normal pooled plasma in the presence of various amounts of emicizumab, respectively. Experiments in all samples were performed at least three separate times and the average values and SEM are shown. Some bars for SEM were shorter than the symbols. CWA, clot waveform analysis; Elg, ellagic acid; TF, tissue factor; CT, clot time; SEM, standard error of mean.

Fig. 3. Thrombin generation in the FXI-deficient plasma in the presence of emicizumab. (A) FXI-deficient plasmas (n = 13) were incubated with various amounts of emicizumab (0 – 100 μg ml^{-1}), followed by measurement of Elg/TF-triggered thrombin generation as described in Methods. A representative dose-dependent thrombogram in one case is illustrated in (A). The obtained parameters,

peak thrombin (B) and endogenous thrombin potentials (ETPs) (C), were plotted as a function of emicizumab in FXI-deficient plasmas. (D) The peak thrombin obtained in TGA in FXI-deficient plasma with various concentrations of purified FXI. The gray zone corresponds to the peak thrombin values obtained in FXI-deficient plasma with emicizumab (100 μg ml^{-1}). The closed circles represent the peak thrombin (B) and ETP (C) values from normal pooled plasma, respectively. Experiments in all samples were performed at least three separate times and the average values and SEM are shown. Some bars for SEM were shorter than the symbols. Elg, ellagic acid; TF, tissue factor; TGA, thrombin generation assay; SEM, standard error of mean.

Fig. 4. Thrombin generation in FVII-deficient or FIX-deficient plasmas with anti-FXI mAb in the presence of emicizumab. FVII-deficient plasma (A) or FIX-deficient plasma (B) that was preincubated with ant-FXI mAb (100 μg ml^{-1}) was mixed with various amounts of emicizumab (0 – 100 μg ml^{-1}), followed by measurement of thrombin generation triggered by Elg/TF as described in Methods. Normal plasma that was preincubated with anti-FXI mAb was regarded as a positive control (FXI-depleted). Experiments were performed using three different batches and representative thrombograms are illustrated in (A) and (B). mAb, monoclonal antibody; Elg, ellagic acid; TF, tissue factor.

Fig. 5. Effects of emicizumab on the FVIIa – FIX association. (A) FIX cleavage; FIX (500 nM) without (upper panel) or with 50 μg ml^{-1} emicizumab (lower panel) was incubated with FVIIa (10 n M)/TF (1 nM) and PL (20 μM) at the indicated times. Samples were analyzed by SDS-PAGE using 8% gels, followed by western blotting with anti-FIX HCh mAb3A6 and anti-mouse peroxidase-linked secondary mAb for detection. Two bands indicated by arrows show the binding of secondary mAb to the heavy and light chains of emicizumab. (B) FVIIa/FIX-catalyzed FXa generation; various concentrations of FIX and PL (20 μM) were reacted with FVIIa/TF (1 n M /0.1 n M). FVIIa reaction was terminated by the addition of E-76 and FXa generation was initiated by the addition of FX (200 nM) as described in Methods. Emicizumab (100 μg ml^{-1}) was added prior to reaction with FVIIa/TF (open circles) or prior to addition of FX (closed circles). Values of FXa generation were plotted as a function of FIX concentration. The experiments were performed on three separate occasions and the average values and SD are shown. Some bars for SD were shorter than the symbols. TF, tissue factor; PL, phospholipid; mAb, monoclonal antibody; SD, standard deviation.

Fig. 6. Thromboelastograms of ROTEM in FXI-deficient whole blood with emicizumab. $CaCl_2$ was added to citrated whole blood from three severe FXI-deficient patients (FXI:C < 1 IU dl^{-1}), to which various amounts of emicizumab were added ex vivo, followed by ROTEM as described in Methods. Representative thromboelastograms in three cases are illustrated in (A) to (C). ROTEM parameters

(CT, CT + CFT) in the patient are shown in each figure. CT and CT + CFT obtained in normal controls (n = 20) were 711 – 1025 and 929 – 1528 s (95% confidence interval), respectively. Experiments were performed two separate times and the representative data are shown. ROTEM, rotational thromboelastometry; CT, clot time; CFT, clot formation time.

Fig. 7. Schema on putative coagulation-enhancing mechanism of emicizumab in FXI-deficient plasma. (A) Emicizumab (–); FVIIa/TF activates the FIX and FX during the initiation phase, followed by the activation of FVIII and FV by a small amount of generated thrombin. Because the absence of FXI does not cause FXIa-catalyzed FIX activation, however, the tenase activity is decreased, resulting in decrease of prothrombinase activity and depression of fibrin formation. (B) Emicizumab (+); FVIIa/TF activates the FIX and FX during the initiation phase, followed by emicizumab-driven FIXa-catalyzed FX activation. In addition, the activated FVIII that is produced by the generated thrombin works as the cofactor on FIXa-catalyzed FX activation. The presence of emicizumab, therefore, enhances the coagulation potentials in the FXI-deficient condition and recovers the decrease of coagulation potentials by the FXI deficiency to a certain extent. TF, tissue factor.

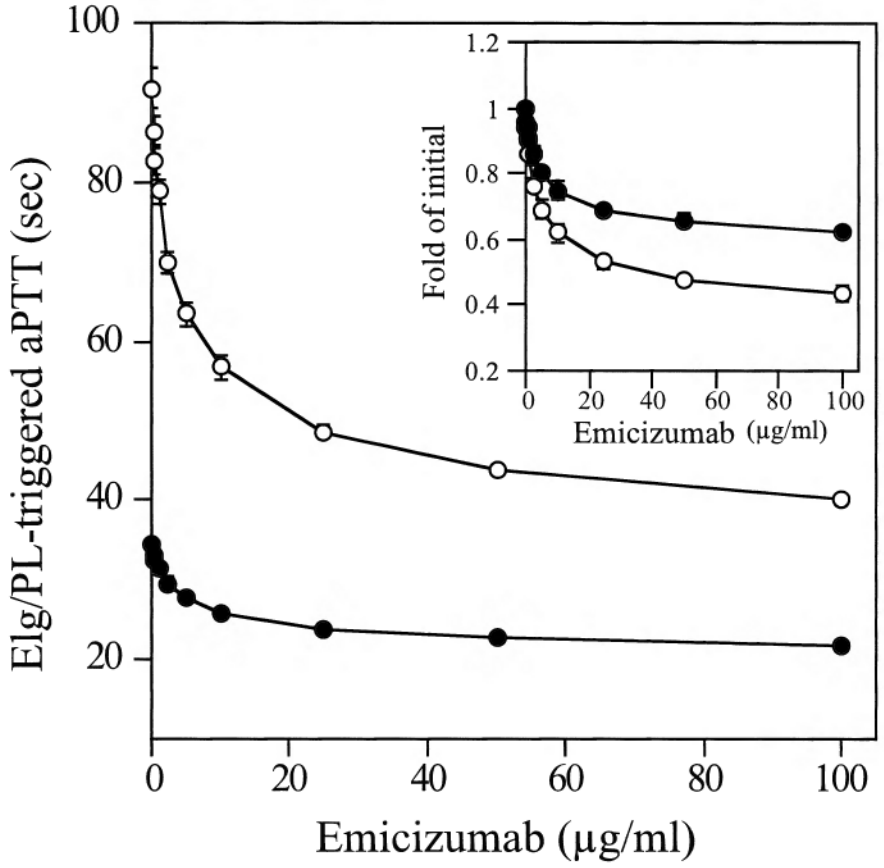
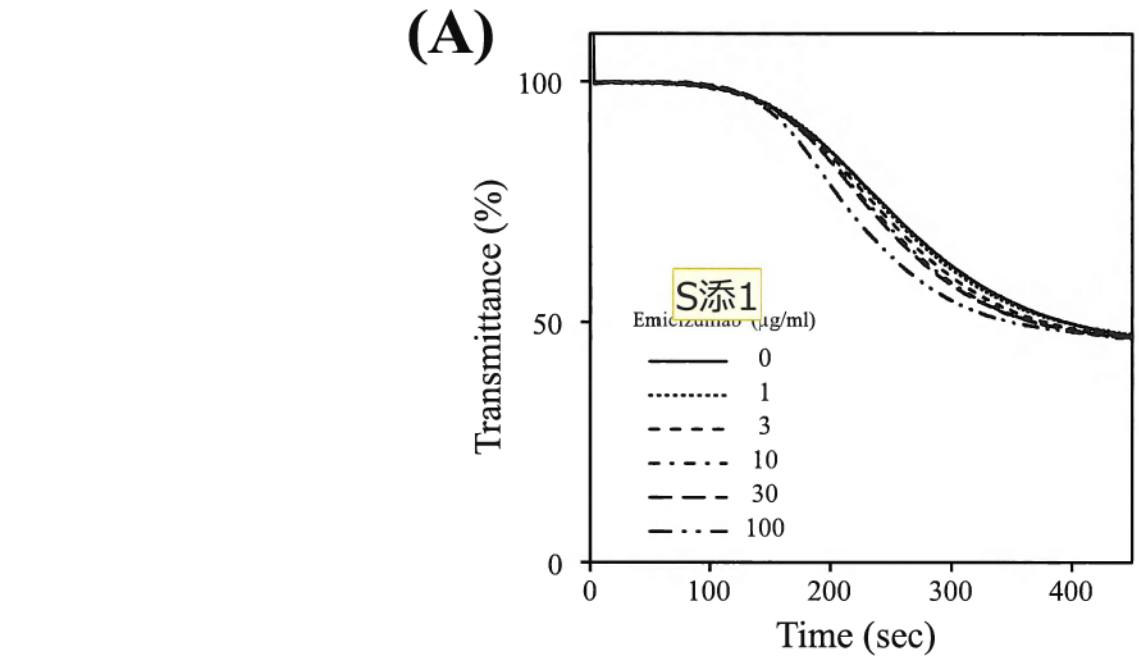
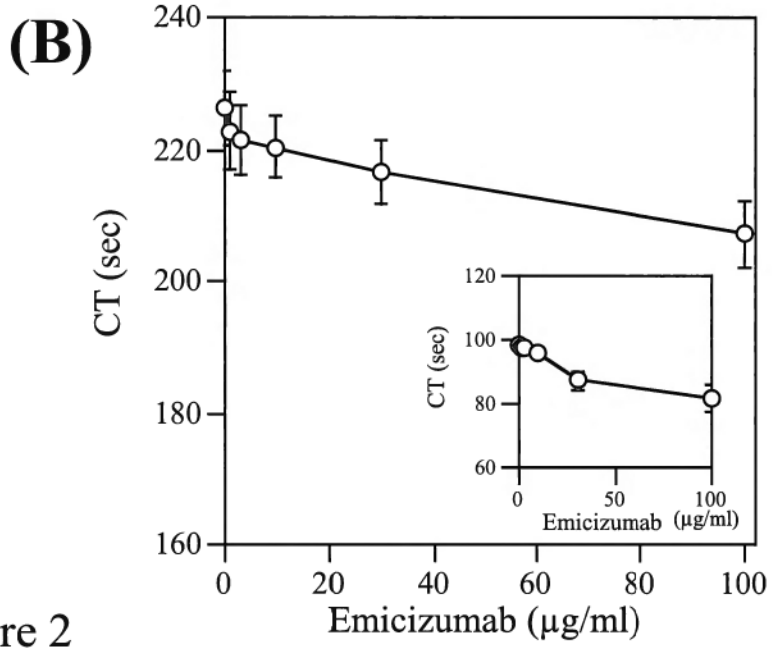

Elg/PL-triggered aPTT (sec)
100
80
60
40
20
0
20
40
60
80
100
Emicizumab (μg/ml)
Fold of initial
1.2
1
0.8
0.6
0.4
0.2
0
20
40
60
80
100
Emicizumab (μg/ml)

Figure 1


(A)
100
50
0
Transmittance (%)
S添1
Emicizumab (μg/ml)
0
1
3
10
30
100
0
100
200
300
400
Time (sec)


(B)
240
220
200
180
160
CT (sec)
120
100
80
60
CT (sec)
0
50
100
Emicizumab (μg/ml)
0
20
40
60
80
100
Emicizumab (μg/ml)

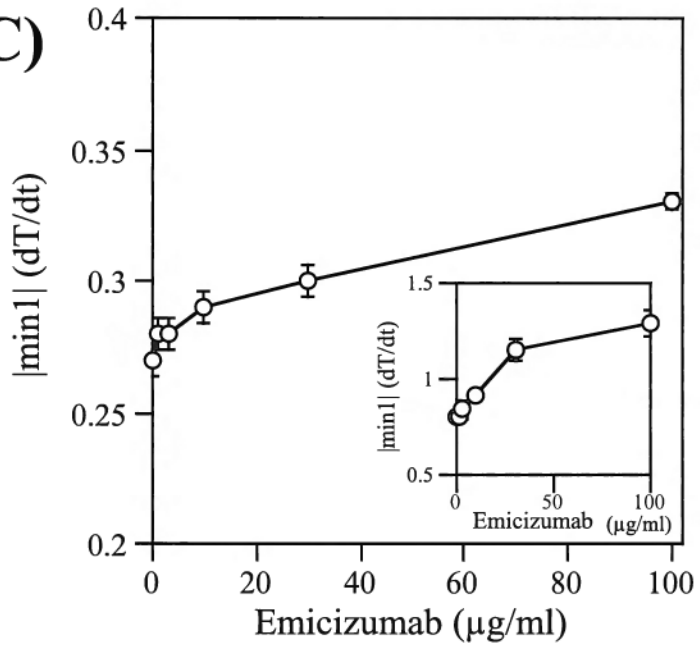

(C)
0.4
0.35
0.3
0.25
0.2
|min1| (dT/dt)
1.5
1
0.5
|min1| (dT/dt)
0
50
100
Emicizumab (μg/ml)
0
20
40
60
80
100
Emicizumab (μg/ml)

Figure 2

スライド 2

S添1　Fig4と同じスタイルがベターと思います。
Soeda,Tetsuhiro 添田哲弘(創薬薬理研究部慢性疾患3G), 2018/08/23

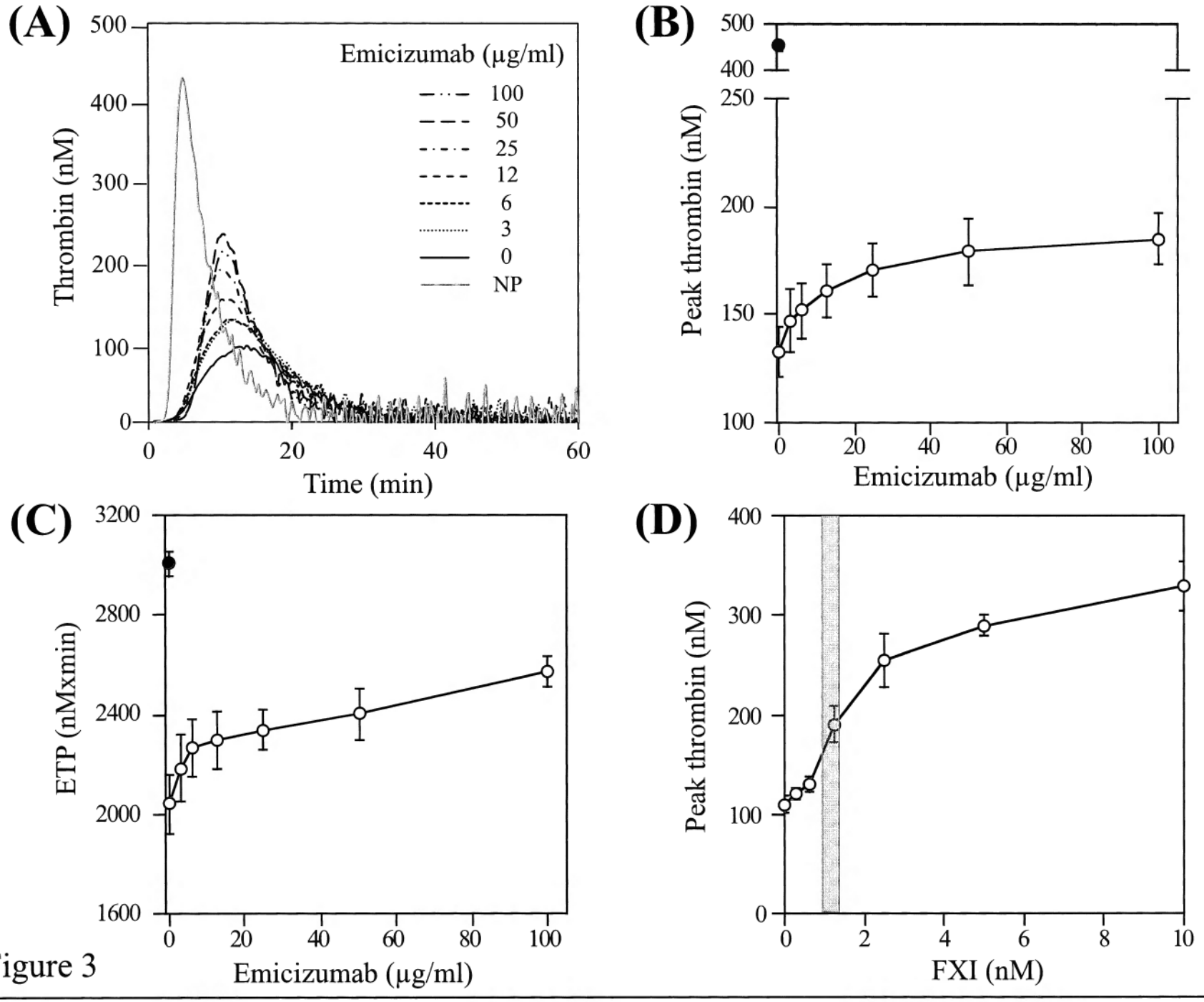

(A)
Emicizumab (µg/ml)
100
50
25
12
6
3
0
NP
Thrombin (nM)
Time (min)
(B)
Peak thrombin (nM)
Emicizumab (µg/ml)
(C)
ETP (nMxmin)
Emicizumab (µg/ml)
(D)
Peak thrombin (nM)
FXI (nM)

Figure 3

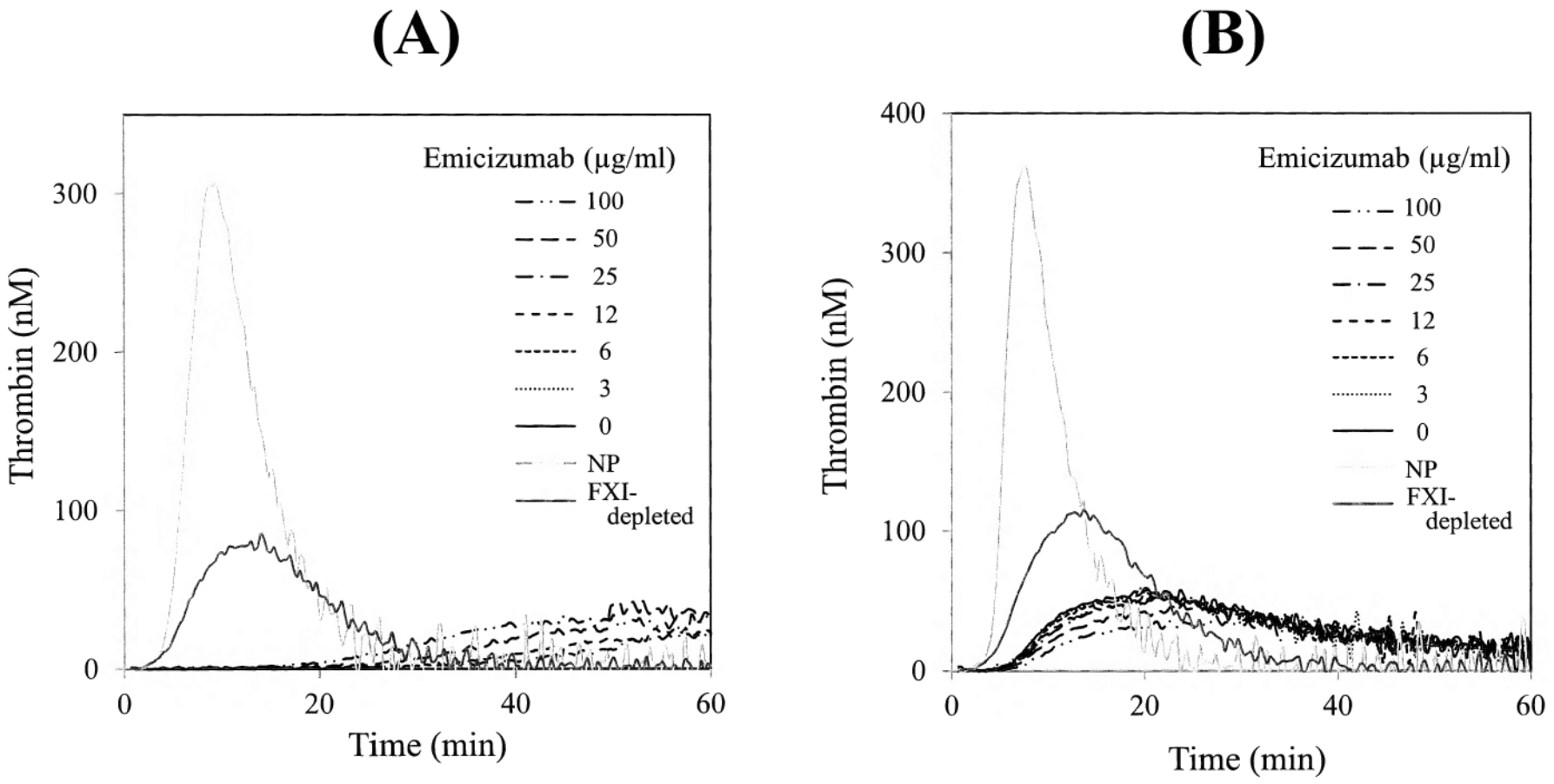

(A)
(B)
Emicizumab (µg/ml)
100
50
25
12
6
3
0
NP
FXI-depleted
Thrombin (nM)
Time (min)
0
100
200
300
400
0
20
40
60

Figure 4

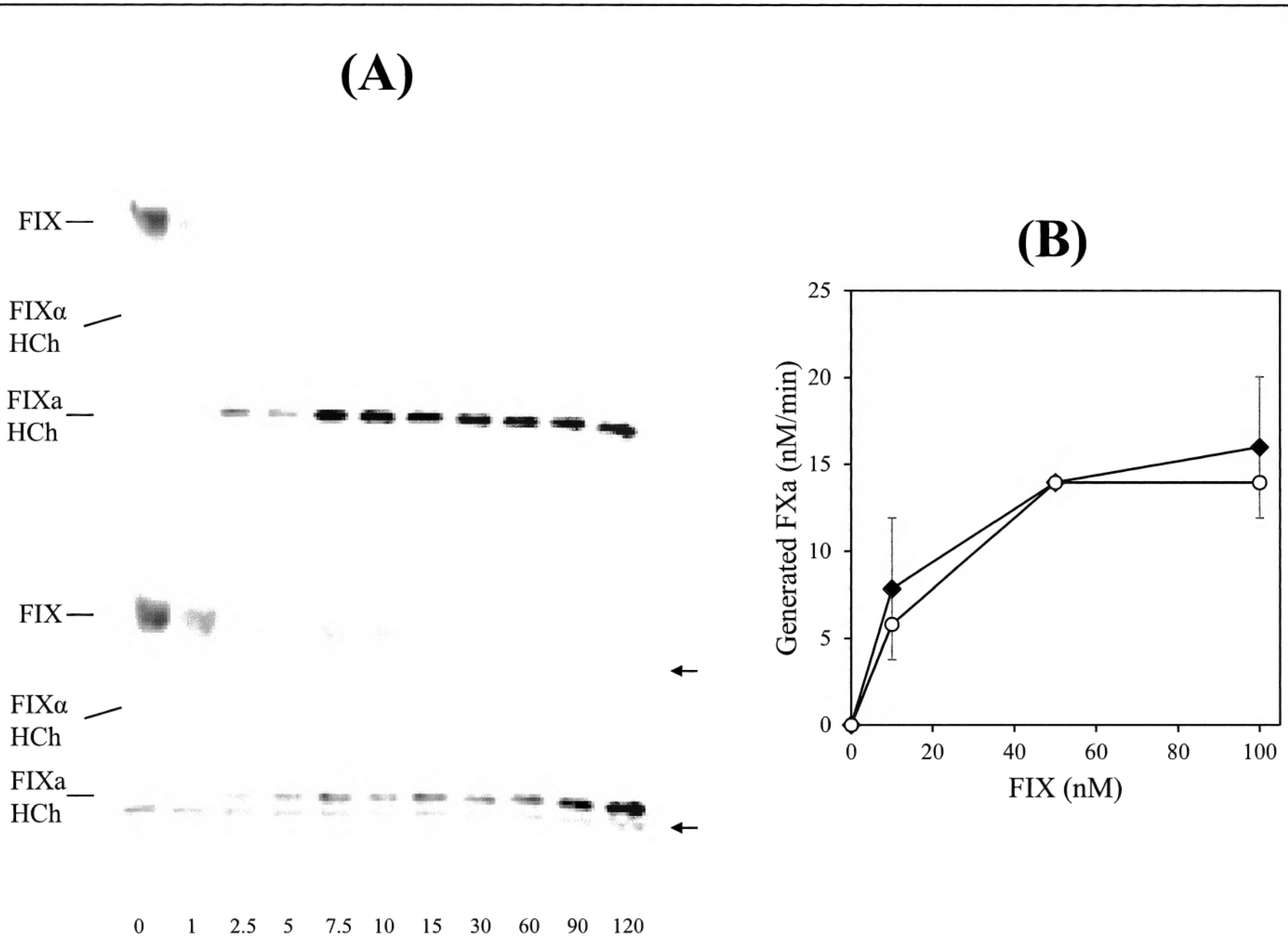

(A)
FIX
FIXα
HCh
FIXa
HCh
FIX
FIXα
HCh
FIXa
HCh
0 1 2.5 5 7.5 10 15 30 60 90 120
(B)
Generated FXa (nM/min)
0 5 10 15 20 25
0 20 40 60 80 100
FIX (nM)

Figure 5

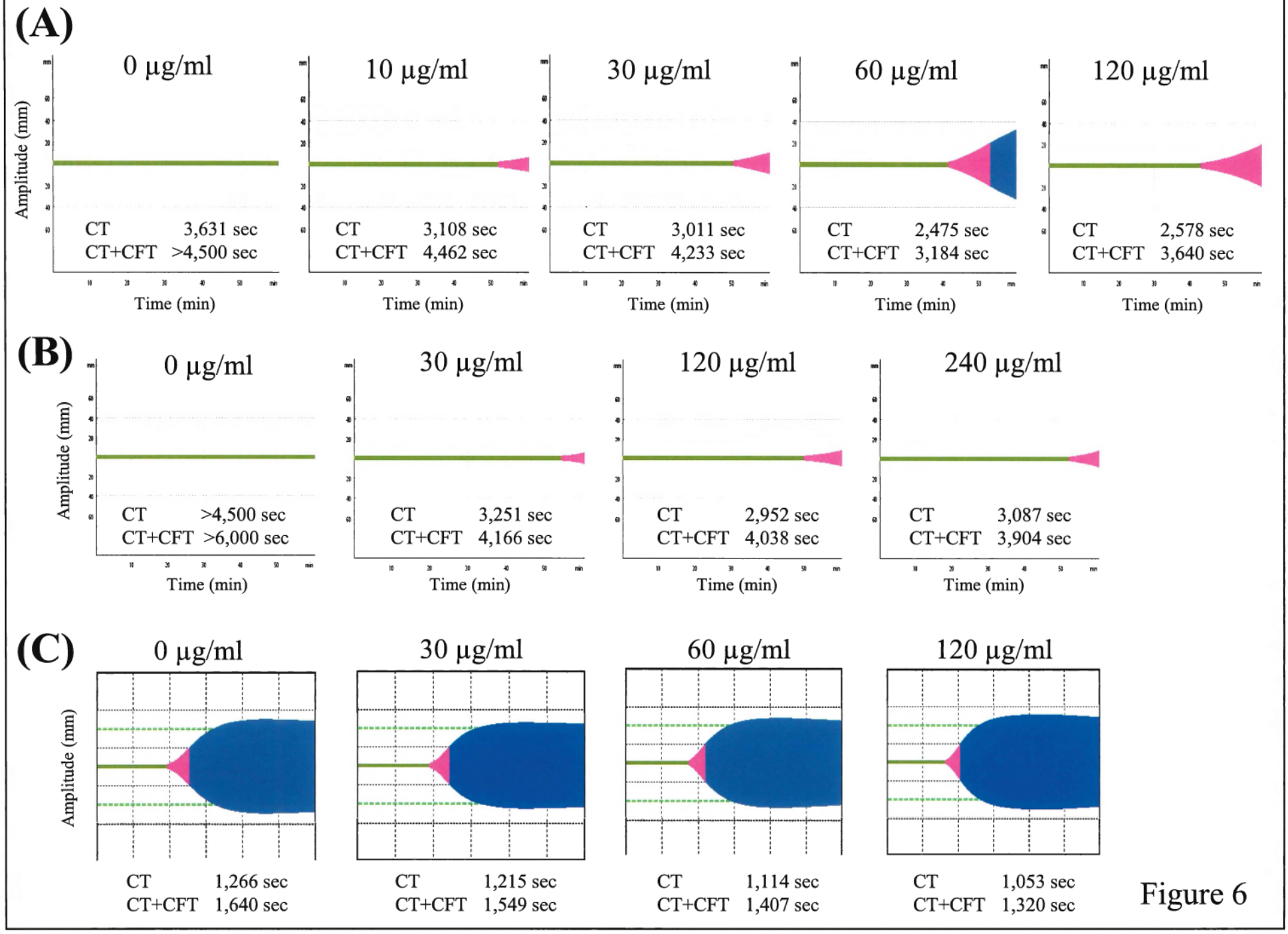

(A)
0 μg/ml
CT 3,631 sec
CT+CFT >4,500 sec
10 μg/ml
CT 3,108 sec
CT+CFT 4,462 sec
30 μg/ml
CT 3,011 sec
CT+CFT 4,233 sec
60 μg/ml
CT 2,475 sec
CT+CFT 3,184 sec
120 μg/ml
CT 2,578 sec
CT+CFT 3,640 sec
Amplitude (mm)
Time (min)
(B)
0 μg/ml
CT >4,500 sec
CT+CFT >6,000 sec
30 μg/ml
CT 3,251 sec
CT+CFT 4,166 sec
120 μg/ml
CT 2,952 sec
CT+CFT 4,038 sec
240 μg/ml
CT 3,087 sec
CT+CFT 3,904 sec
(C)
0 μg/ml
CT 1,266 sec
CT+CFT 1,640 sec
30 μg/ml
CT 1,215 sec
CT+CFT 1,549 sec
60 μg/ml
CT 1,114 sec
CT+CFT 1,407 sec
120 μg/ml
CT 1,053 sec
CT+CFT 1,320 sec

Figure 6

S添2

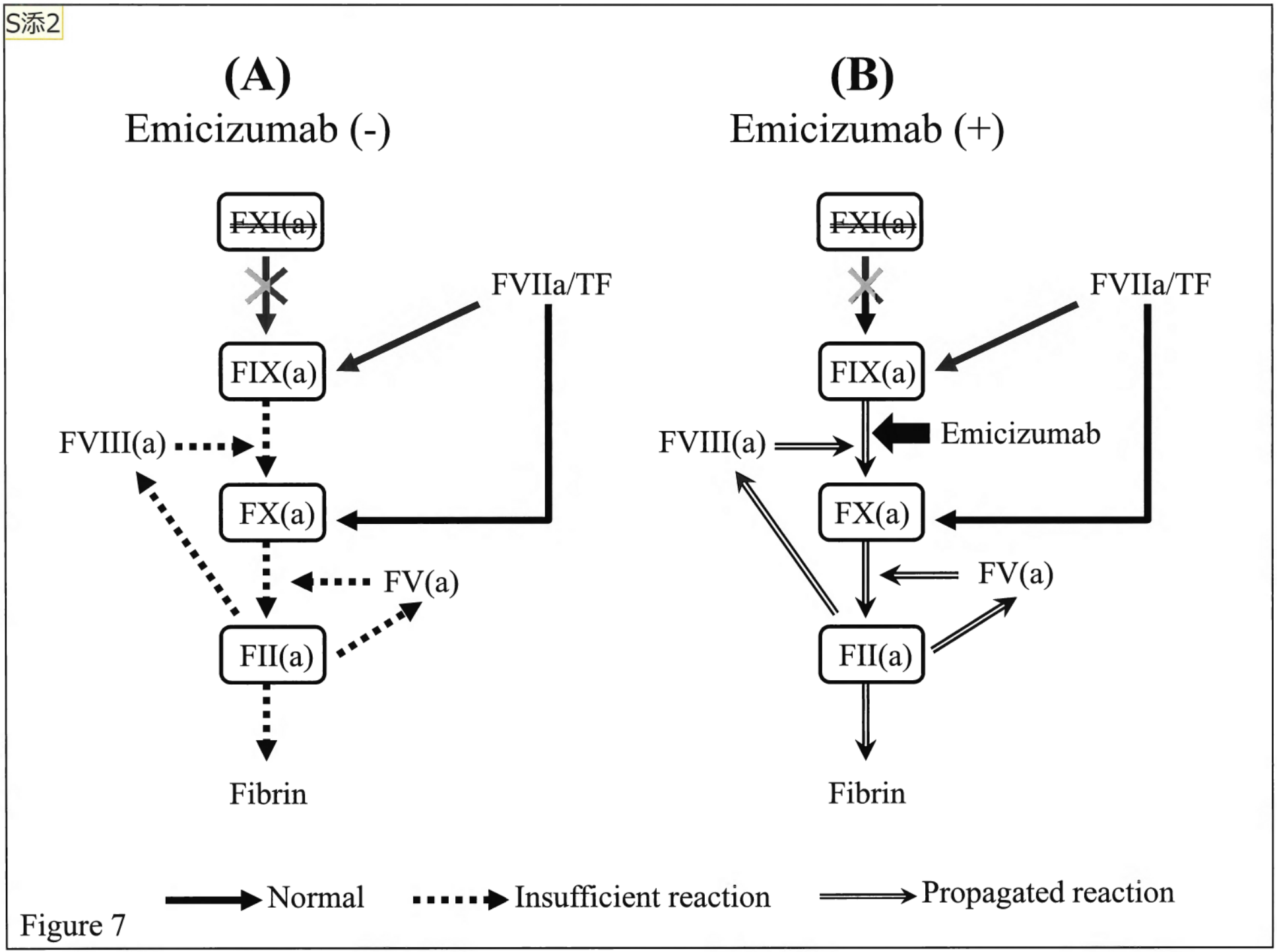
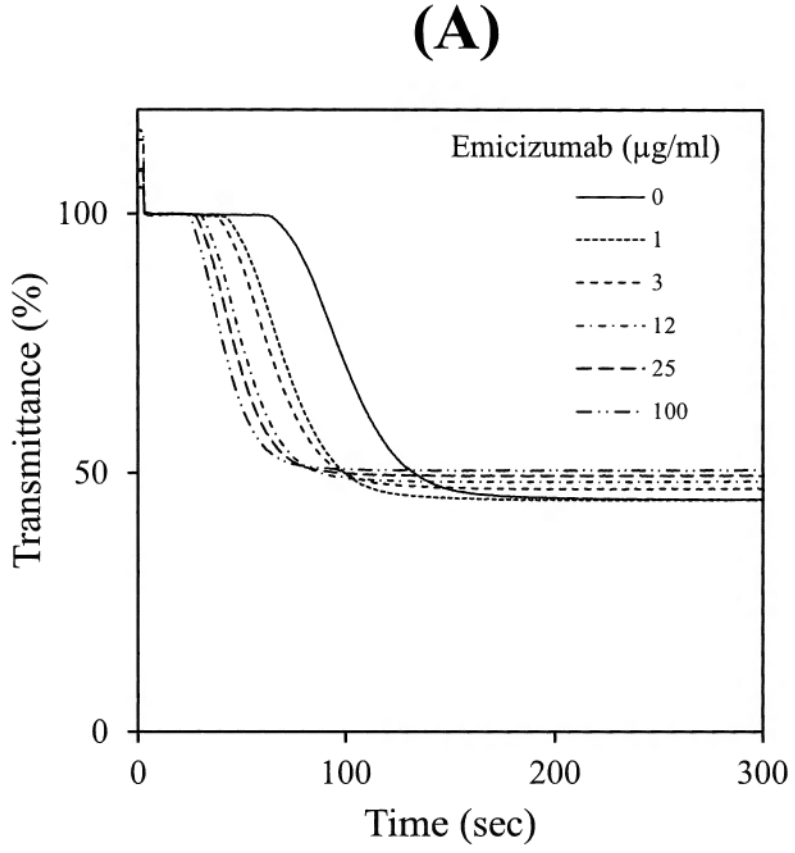
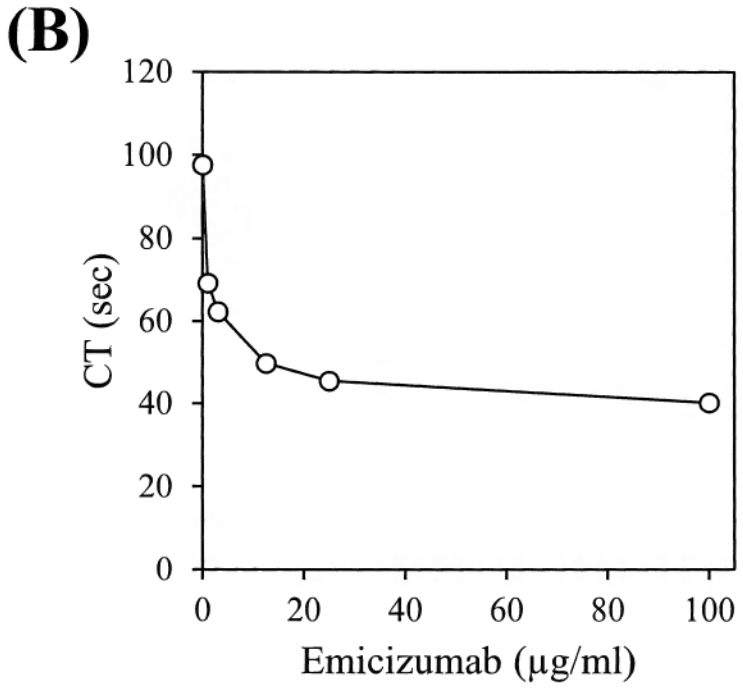

(A)
Emicizumab (-)
FXI(a)
FVIIa/TF
FIX(a)
FVIII(a)
FX(a)
FV(a)
FII(a)
Fibrin
(B)
Emicizumab (+)
FXI(a)
FVIIa/TF
FIX(a)
Emicizumab
FVIII(a)
FX(a)
FV(a)
FII(a)
Fibrin
Normal
Insufficient reaction
Propagated reaction

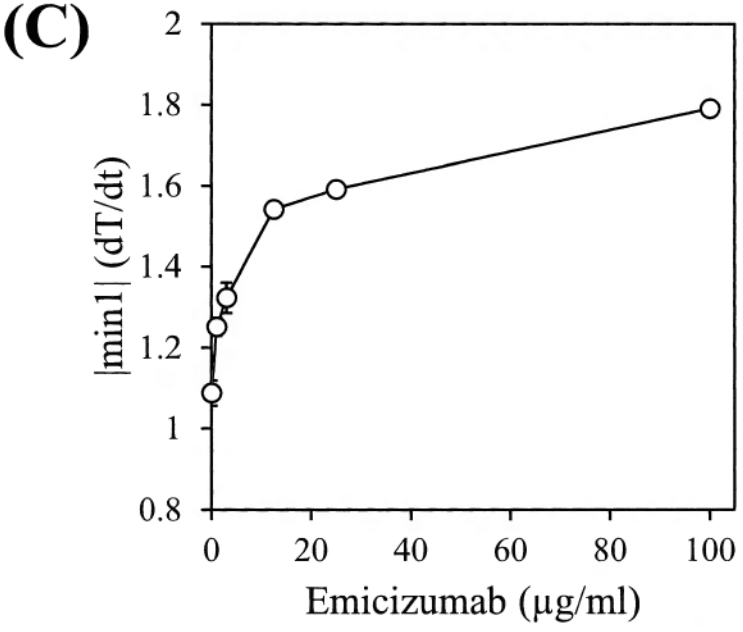
Figure 7

スライド 7

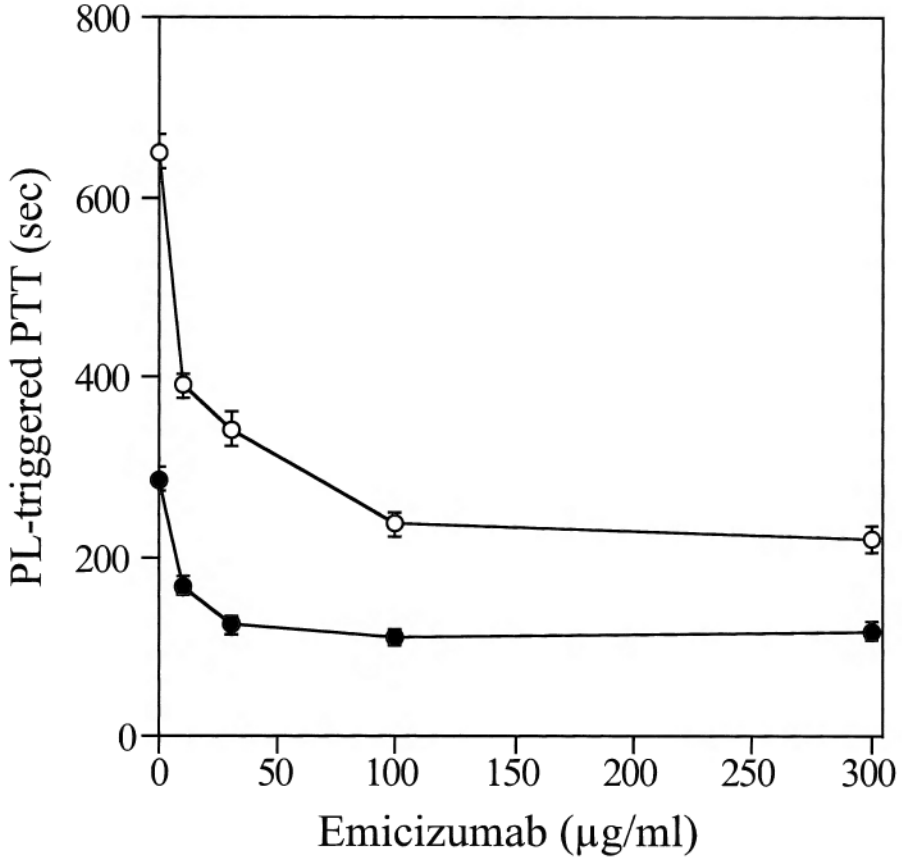
S添2　添田：図7につきまして、北沢と再度確認し、絵の矢印の修正、表現を修正致しました。
Soeda,Tetsuhiro 添田哲弘(創薬薬理研究部慢性疾患3G), 2018/10/12







Supplemental data S1



Supplemental data S2