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Hemostatic Treatment Using Factor VIII Concentrates for Neutralizing High-Responding Inhibitors Prior to CVAD Insertion for Immune-Tolerance Induction Therapy

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Abstract

We have utilized high-dose factor VIII (FVIII) concentrates in 4 hemophilia A patients with inhibitors prior to surgery for the insertion of a central venous access device. In total, 3 patients out of 4 had high responding inhibitors. Dosing algorithms for this type of therapy have not been previously validated and established. We devised an effective formula to calculate the initial dose required to neutralize the inhibitors, although some of the patients demonstrated a lower recovery of FVIII than expected. An anamnestic inhibitor response was evident in 3 cases, but overall our strategy provided a reliable hemostatic effect for at least 4 days after surgery. In addition, our protocol appeared to be more cost-effective than FVIII bypass therapy. The financial saving in 1 case for the initial 3 days was estimated to be approximately US$49 122. Our results demonstrated that high-dose FVIII therapy provided clinically effective and economically viable results even in high responders.

Keywords

hemophilia, inhibitor, CVAD, high-dose factor VIII, cost-effectiveness

Introduction

Hemophilia A is the most common X-linked hereditary hemorrhagic disorder and is caused by a deficiency or functional abnormality of blood clotting factor VIII (FVIII). Bleeding episodes are commonly treated by infusions of plasma-derived FVIII (pdFVIII) concentrates or recombinant FVIII (rFVIII) concentrates. Factor VIII inhibitors arise as alloantibodies against infused, extrinsic FVIII in 15% to 30% of patients with hemophilia A.[1] In these instances, the hemostatic efficiency of infused FVIII is seriously impaired, and the development of alloantibodies, therefore, represents a serious therapeutic complication in such patients. Patients with FVIII inhibitors can be classified into 2 subgroups, high and low responders. High responders have inhibitor levels of >5 BU/mL and exhibit a brisk antibody response following FVIII exposure. The inhibitor titer in these cases usually increases sharply 5 to 7 days after an infusion of FVIII, and this phenomenon is believed to be an anamnestic response. Low responders have inhibitor levels of <5 BU/mL and do not develop an anamnestic response following exposure to FVIII.[2,3] Factor VIII-bypassing agents such as activated prothrombin complex concentrates (aPCC) and recombinant activated factor VIIa (rFVIIa) are commonly used for the treatment of hemarthroses in high responders. Inhibitor titers do not remain constant in patients with hemophilia A, however, and after long-term absence of a FVIII antigenic stimulus, inhibitor titers often decrease to the levels where high-dose FVIII concentrate therapy may be used even in high responders. In this study, we utilized high-dose FVIII concentrates in 4 hemophilia A patients with inhibitors prior to surgery for the insertion of a central venous access device (CVAD). All patients were considered suitable for immune tolerance induction (ITI) therapy through the implanted device. There appears to be few reports in which practical strategies for the use of

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Table 1. Profiles of 4 Hemophilia A Patients With Inhibitor and Infused Doses of FVIII Concentrate[a]

	Case 1	Case 2	Case 3	Case 4
Age at the CVAD insertion	2Y9M	6Y11M	7Y7M	1Y2M
Maximum inhibitor titer recorded, BU/mL	24.0	204	24.7	3.0
BW, kg	13.0	18.0	24.0	8.9
Hematocrit, %	40.9	34.1	25.8	39.2
Inhibitor titer at the CVAD insertion, BU/mL	6.8	6.0	2.9	2.4
Dose of infusion, U	3000	4000	3500	1000
Expected FVIII:C, %	140	128	120	108
Observed FVIII:C, %	88	9	52	80
Continuous doses in need of maintaining, U/kg/h	7.7-15.4	8.9-22.2	5.0-8.3	5.6-8.4

Abbreviations: CVAD, central venous access device; FVIII, factor VIII; Y, year; M, month.

[a] The dose (U) to neutralize the inhibitor was calculated according to the equation: FVIII dose to neutralize inhibitors = 80 × BW (kg) × {100 − Ht(%)} × 100 − 1 × inhibiter titer (BU/mL) × 0.5. An additional 54 to 70 U/kg FVIII concentrate was added and administrated to each patient. FVIII coagulant activity (FVIII:C) was monitored 30 minutes after the bolus infusion. Both expected and observed FVIII:C were represented for each case. After confirmation that FVIII:C level was satisfactory, continuous infusion of FVIII concentrate was administered to maintain the therapeutic FVIII:C level. Case 2 required additional 2000 U as bolus infusions before maintaining therapy.

high-dose FVIII concentrates in high responders have been described. We demonstrated that the neutralization of high-responding inhibitors can be hemostatically effective. We described retrospective case reports of surgery performed in these patients between 2006 and 2010.

Materials and Methods

Patient Profiles

All patients had severe hemophilia A with inhibitors (FVIII coagulant activity [FVIII:C], <1%) and were considered suitable for ITI. In each case, however, the current venous access was believed to be too challenging for the necessary frequent infusions, and CVAD placement was planned to facilitate the program. The clinical and laboratory profiles of these patients are summarized in Table 1.

Case 1: Patient was 2 years old and was diagnosed at the age of 3 months. Subsequently, he was treated with pdFVIII on demand for recurrent hemorrhagic episodes. At the age of 15 months, a FVIII inhibitor was detected (1.0 BU/mL). Since then he has had repeated muscle and joint bleeding, and although he has been treated with FVIII-bypassing agents, he developed progressive hemophilic arthropathy. The maximum inhibitor titer recorded (highest inhibitor level since diagnosis) was 24 BU/mL which decreased to 6.8 BU/mL at the time of surgery.

Case 2: Patient was 6 years old. He was diagnosed at the age of 5 months. At the age of 10 months, he was admitted with an intractable hemorrhage in the scrotal sack, and a FVIII inhibitor (80 BU/mL) was detected. Since then he has had repeated muscle and joint bleeding, and although he has been treated with bypassing agents, both knee joints have been destroyed. He has difficulty in walking unaided and is usually confined to a wheelchair. The maximum inhibitor titer recorded was 204 BU/mL which decreased to 6.0 BU/mL at the time of surgery.

Case 3: Patient was 7 years old. He was the younger brother of case 2 and was diagnosed at the age of 1 month. At the age of 1 year, a FVIII inhibitor was detected. As with his older brother, he has been treated with bypassing agents but has had repeated muscle and joint bleeding and developed progressive hemophilic arthropathy. The maximum inhibitor titer was 24.7 BU/mL, which decreased to 2.9 BU/mL at the time of surgery.

Case 4: Patient was 1 year old and was diagnosed at the age of 2 months. At the age of 9 months, a FVIII inhibitor (1.0 BU/mL) was detected. His family requested ITI instead of using bypassing agents to prevent serious joint deterioration. Difficulties with venous access prompted us to consider the insertion of a CVAD. The maximum inhibitor titer was 3.0 BU/mL which decreased to 2.4 BU/mL at the time of surgery.

Protocol

All therapy was conducted after obtaining fully informed consent. Before the start of surgery, in each case sufficient high-dose FVIII concentrate was administrated to overcome the circulating inhibitor. Previous dosing algorithms for this type of procedure have little scientific basis, however, and have not been validated[2]; and in the absence of a rational and validated approach, we devised the following formula to estimate the amount of FVIII required to neutralize the inhibitors. FVIII dose = 80 × body weight BW (kg) × {100 − Ht(%)} × 100^{-1} × inhibiter titer (BU/mL) × 0.5, where the calculations incorporating the body weight (BW) in kilogram and the hematocrit (Ht%) provided an estimate of the circulating plasma volume (mL). An additional 50 U/kg FVIII concentrate was added to obtain the therapeutic dose, and the resulting circulating FVIII:C was expected to be 100% (1.0 U/mL). After confirmation that the FVIII:C level was satisfactory, continuous infusion of FVIII concentrate was administered to maintain therapeutic levels. FVIII:C was monitored during continuous infusion, with target levels of FVIII:C above 50% (0.5 U/mL) for 2 days after the surgery, and above 20% (0.2 U/mL) for a further 3 to 5 days. Immune tolerance induction therapy was commenced 7 to

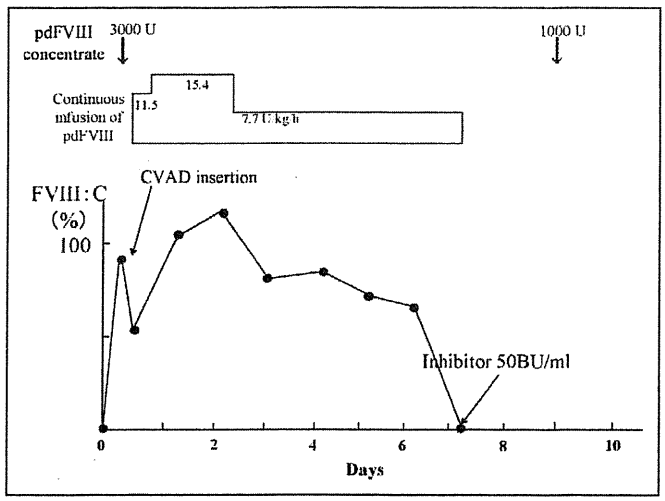

Figure 1. Clinical course of case 1. The FVIII:C after the infusion of 3000 U pdFVIII was 88% (expected 140%). Subsequently, continuous infusion of FVIII concentrate was administered. The patient required the dose of 7.7 to 15.4 U/kg per h to maintain the target levels of circulating FVIII:C. Seven days later, the inhibitor level was elevated to 50 BU/mL. pdFVIII indicates plasma-derived factor VIII; FVIII:C, FVIII coagulant activity.

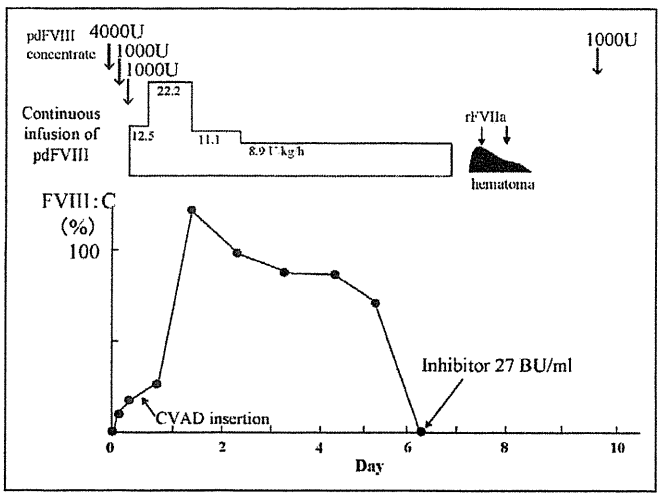

Figure 2. Clinical course of case 2. The FVIII:C in case 2 after the infusion of 3000 U pdFVIII was only 9.4% (expected 128%). An additional 1000 U pdFVIII raised the FVIII:C to 16%. A further 1000 U pdFVIII was given and surgery performed immediately. The operation was completed without untoward bleeding. Subsequently, continuous infusion of pdFVIII was administered. The patient required the higher doses of FVIII (8.9-22.2 U/kg per h) than anticipated to maintain the therapeutic level of FVIII:C. Six days after surgery, a circulating inhibitor was detected at a titer of 27 BU/mL in the absence of FVIII:C. pdFVIII indicates plasma-derived factor VIII; FVIII:C, FVIII coagulant activity.

10 days later using 50 U/kg FVIII concentrate 3 times a week administered through the implanted devices. Standard, 5F port catheters (Anthron P-U Catheter, TORAY Medical Co Ltd, Tokyo, Japan) were utilized as CVADs in all cases. The devices consisted of a subcutaneous reservoir with a self-sealing silicone septum coupled to a radiopaque silicone catheter. They were implanted as generally accepted in the upper anterior chest wall with the catheter fed via the jugular venous system into the superior vena cava (SVC) such that the catheter tip was located at or just above the entry of the SVC into the right atrium. Port access was gained using a specialized noncoring percutaneous needle (Huber needle, B. Braun Medical Inc, Bethlehem, Pennsylvania).[4]

Factor VIII Assays

FVIII:C was measured using 1-stage procoagulant assays as described previously.[5] Factor VIII inhibitor assays were performed using the Bethesda method.[6]

Results

The doses of FVIII concentrate administrated and FVIII:C recovery are summarized in Table 1. The clinical course in each case is shown in Figures 1 to 4, respectively.

The plasma level of FVIII:C in case 1 after the infusion of 3000 U pdFVIII (Cross-Eight M, The Japanese Red Cross, Chitose, Japan) was 88% (expected 140%). Subsequently, continuous infusion of FVIII concentrate was administered at a dose of 7.7 to 15.4 U/kg per h to maintain the target levels of circulating FVIII:C. Surgery was completed without significant blood loss, and no bleeding was observed after the operation.

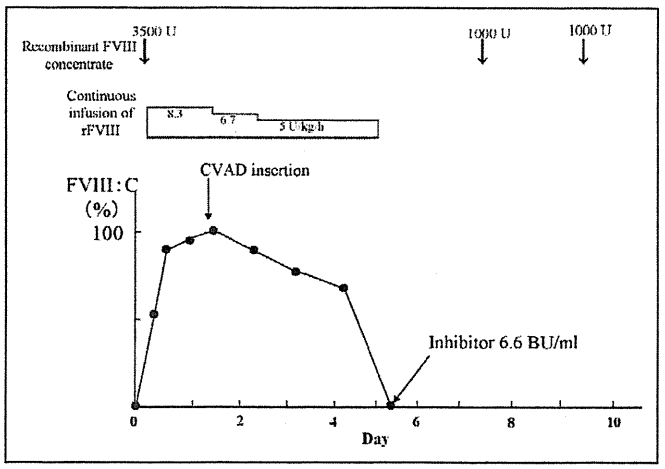

Figure 3. Clinical course of case 3.
The plasma level of FVIII:C in case 3 after the infusion of 3500 U rFVIII was 52% (expected 120%). The bolus infusion was followed by continuous infusion of at a dose of 8.3 U/kg per h and 12 hours later the plasma level of FVIII:C was 90%. Central venous access device insertion was finished without significant blood loss. Continuous infusion of rFVIII was administered at a dose of 5 to 8.3 U/kg per h to maintain the target levels of FVIII:C. No intra and postoperative bleeding was observed. An anamnestic response stimulated the inhibitor to 50 BU/mL 5 days after the initiation of high-dose FVIII therapy. FVIII:C indicates factor VIII coagulant activity; rFVIII, recombinant FVIII.

Seven days later, an anamnestic response stimulated the inhibitor level to 50 BU/mL, and no FVIII:C was present in plasma. The inhibitor level declined after ITI therapy and a normal

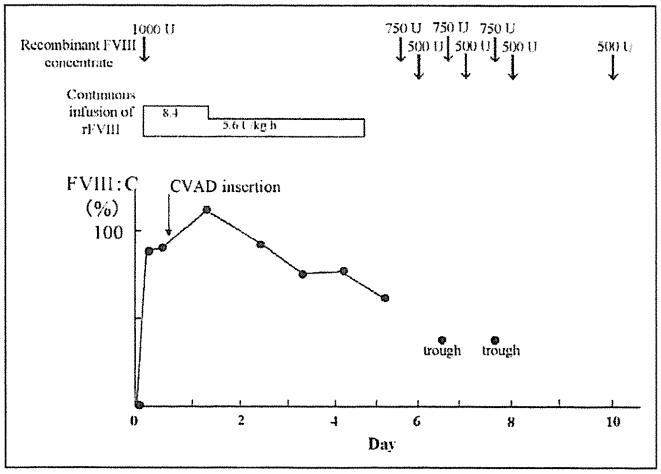

Figure 4. *Clinical course of case 4.*
The plasma level of FVIII:C in case 4 after the infusion of 1000 U rFVIII was 80% (expected 109%). The bolus infusion was followed by continuous infusion at a dose of 8.4 U/kg per h. Twelve hours later, the plasma level of FVIII:C was 90%, and the surgery commenced. Central venous access device insertion was completed without significant blood loss. Continuous infusion of rFVIII was administered at a dose of 5.6 to 8.4 U/kg per h to maintain the target levels of FVIII:C. No postoperative bleeding was observed. Ten days after surgery, the inhibitor titer rose to 1.8 BU/mL. FVIII:C indicates factor VIII coagulant activity; rFVIII, recombinant FVIII.

FVIII half-life and complete disappearance of inhibitor was confirmed 2½ years later. Clinical hemorrhagic events decreased dramatically and the patient has overcome serious joint problems. The CVAD was removed 3 years after the surgery. No complications were observed during the period of the implantation.

In contrast, the plasma level of FVIII:C in case 2 after the infusion of 3000 U pdFVIII (Cross-Eight M) was only 9.4% (expected 128%). An additional 1000 U pdFVIII raised the FVIII:C to 16%. A further 1000 U pdFVIII was given and the surgery was performed immediately. The operation was completed without untoward bleeding. Subsequently, continuous infusion of pdFVIII at a dose of 8.9 to 22.2 U/kg per h was administered to maintain the target levels of FVIII:C. The presence of the latent inhibitor required the use of higher doses of FVIII than anticipated to maintain the therapeutic level of FVIII:C. Six days after surgery, a circulating inhibitor was detected at a titer of 27 BU/mL in the absence of FVIII:C. A hematoma developed around the implanted reservoir 7 days after the surgery but resolved in a few days after treatment with rFVIIa. Subsequently, ITI therapy was attempted for 2 years, and although the inhibitor titer declined to 1.7 BU/mL 60 weeks after surgery, it was later elevated to 103 BU/mL and ITI therapy was discontinued. The patient continues to have repeated joint and muscle hemorrhages.

The plasma level of FVIII:C in case 3 after the infusion of 3500 U rFVIII (Advate, Baxter Healthcare Corporation, Neuchatel, Switzerland) was 52% (expected 120%). The bolus infusion was followed by continuous infusion at a dose of 8.3 U/kg per h and 12 hours later the plasma level of FVIII:C was 90%. Central venous access device insertion was accomplished without significant blood loss. Continuous infusion of rFVIII was administered at a dose of 5 to 8.3 U/kg per h to maintain the target levels of FVIII:C. No intra or postoperative bleeding was observed. An anamnestic response stimulated the inhibitor to 50 BU/mL 5 days after the initiation of high-dose FVIII therapy. Immune tolerance induction therapy has been continued for 1½ years; and although the inhibitor titer reached a peak of 385 BU/mL, it has presently declined to 37 BU/mL.

The plasma level of FVIII:C in case 4 after the infusion of 1000 U rFVIII (Advate) was 80% (expected 109%). The bolus infusion was followed by continuous infusion at a dose of 8.4 U/kg per h. Twelve hours later, the plasma level of FVIII:C was 90%, and the surgery commenced. Central venous access device insertion was completed without significant blood loss. Continuous infusion of rFVIII was administered at a dose of 5.6 to 8.4 U/kg per h to maintain the target levels of FVIII:C. No postoperative bleeding was observed. Ten days after the surgery, the inhibitor titer rose to a maximum of 1.8 BU/mL, consistent with the behavior of a low-responding inhibitor. Immune tolerance induction therapy has been continued for 5 months and the inhibitor titer currently remains at 1.2 BU/mL.

Discussion

High-dose FVIII therapy provided reliable hemostatic control and successfully prevented intra and postoperative hemorrhage in each of the present case, although 3 of the 4 patients had high responding inhibitors. Assessment of the effectiveness of FVIII-bypassing therapy for controlling hemostasis in hemophilia A patients with inhibitors is known to be difficult. Thromboelastgraphy (TEG)[7] and thrombin generation assays (TGT)[8] have been utilized for this purpose, but these techniques are not widely adopted and some of the assay parameters can be difficult to interpret. In addition, long-term use of aPCC or rFVIIa might be refractory in some cases.[9] In contrast, high-dose FVIII therapy appears to be hemostatically effective once the circulating inhibitor is neutralized. In the present study, anamnestic responses were evident in cases 1 to 3, but therapeutic levels of FVIII:C were maintained above 20% for 5 days after commencing high-dose FVIII therapy. After starting the therapy, the time when the antibody response was seen varied between patients (days 7, 6, and 5 in cases 1-3, respectively), indicating that careful monitoring of FVIII:C was indispensable for reliable clinical management.

Importantly, our experience demonstrated that mathematical calculations of the required FVIII dose based on the inhibitor titer and blood volume may not be reliable. In case 2 especially, much more exogenous FVIII was required than calculated in order to neutralize the inhibitor and maintain therapeutic FVIII:C levels. Previous studies have suggested that inhibitors may be contained in the so-called third, extravascular space, including interstitial fluid, lymph nodes, and splenic tissue.[10] Although inhibitors may be present in interstitial fluid in equilibrium with plasma, it is difficult to predict the amount of antibodies in the third space in any individual patient. Our equation, FVIII doses to neutralize inhibitors = (80 × BW

Table 2. Economical Comparison[a] Between High-Dose FVIII Therapy and FVIIa Bypassing Therapy in Case 3

	Recombinant FVIII Concentrate		Recombinant FVIIa	
	Usage, U	Expense, JPY (USD)	Usage, mg	Expense, JPY(USD)
Preoperation	7000	517 069 (5560)	2.4	233 002 (2505)
0-24 Hours after operation	4000	295 468 (3177)	19.2	1 864 016 (20 040)
24-48 Hours after operation	3000	221 601 (2382)	19.2	1 864 016 (20 040)
48-72 Hours after operation	3000	221 601 (2382)	19.2	1 864 016 (20 040)
Total	17000	1 255 739 (13 503)	60	5 825 050 (62 625)

Abbreviations: FVIII, factor VIII; JPY, Japanese yen.

[a] The financial implications of high-dose FVIII replacement compared with FVIII-bypassing therapy are represented by case 3. On the basis of current recommendations, rFVIIa would be given every 3 hours for the initial 3 days.

(kg) × {100–Ht(%)} × 100^{-1} × inhibiter titer (BU/mL)] × 0.5), excludes noncirculating inhibitor and might underestimate the required dose in some circumstances. Kasper proposed an alternative equation, {40 × BW (kg) + 20 × BW (kg) × inhibiter titer (BU/mL)}, to determine the initial dose,[11] but using this method in our cases indicated that 2288, 2880, 2352, and 783 U should be used for cases 1, 2, 3, and 4, respectively. These amounts were much lower than those that were eventually required. Furthermore, recalculation[12] using van Leuven's equation {2 × BW (kg) × inhibiter titer (BU/mL) × 0.8 × {100 − Ht(%)} + 50 × BW (kg)}, overestimated the dose (9009, 12 287, 8038, and 2523 U for cases 1, 2, 3, and 4, respectively). It seems likely, therefore, that no formula can be reliably applied to all cases, although our equation provided the closest estimate to that required in 3 of our patients. Our findings in case 2 also indicated that adequate time should be allowed before surgery to ensure FVIII inhibitor neutralization. Infusions of high doses of FVIII concentrate were commenced 12 hours before the surgery in cases 3 and 4; and in these instances, the inhibitors were neutralized and effective levels of FVIII:C were established. This protocol ensured adequate hemostasis without fear of sudden FVIII loss during the course of surgery.

Our findings do not infer that this type of treatment maximized FVIII recovery or that ITI was successful in all cases, but our data suggest that the strategy of using FVIII concentrate successfully provided a reliable hemostatic effect for at least 4 days after surgery. The hematoma that appeared in case 2 was not unusual and is seen often in patients with hemophilia at the site of the self-sealing silicone septum after establishing port access with a percutaneous needle. The hematoma was not regarded as serious and disappeared quickly after treatment with rFVIIa. Generally, the clearance of FVIII is known to be 2 to 4 mL/kg per h, and infusions of pdFVIII or rFVIII at 3 U/kg per h is usually enough to maintain therapeutic levels in patients without inhibitors.[13] In our cases with inhibitors, especially cases 1 and 2, much higher doses of continuous pdFVIII were required, and although the clearance rates are known to differ between individuals, it seems likely that our results in cases 1 and 2 were associated with inhibitor-mediated enhanced clearance of FVIII. Further studies are required to clarify the effects of inhibitors on clearance of infused FVIII. Nevertheless, overall our results clearly illustrated that plasma FVIII:C should be carefully monitored during and after concentrate infusion so that additional material can be administrated if the therapeutic levels are not adequate.

In general, treatment costs for patients with inhibitors are usually considerably higher than those for patients without inhibitors.[14] The financial implications of high-dose FVIII replacement compared with FVIII-bypassing therapy are represented by case 3 and illustrated in Table 2. Hemostatic control for the initial 3 days after surgery is especially important, and on the basis of current recommendations,[15] rFVIIa (NovoSeven, Novo Nordisk, Bagsvaerd, Denmark) would be given at a dose of 80 to 120 μg/kg every 3 hours for this period. For this reason, therefore, we compared these therapeutic options for this period. Hence, the financial saving in this case for this time would be 4.6 million yen (JPY), approximately US$49 122. Similar economic benefits would be seen in the other 3 cases. The financial savings were calculated to be US$20 988, US$44 357, and US$26 547 in cases 1, 2, and 4, respectively. The data indicate, therefore, that high-dose FVIII therapy could be substantially more cost-effective than bypassing therapy in these circumstances.

With regard to the efficacy of ITI in our patients, case 1 was successful, whereas case 2 failed. Therapy is still ongoing in cases 3 and 4. The importance of the intensity of initial therapy on the outcome of ITI remains to be fully evaluated. Nevertheless, although minor surgery such as CVAD insertion could be performed under bypassing therapy, our results emphasize that high-dose FVIII therapy is clinically effective and economically viable in patients with high-responding inhibitors. In addition, our findings suggest that this hemostatic strategy could be applicable to more invasive operations in patients with hemophilia A.

Authors' Note

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Factor VIII activation by factor VIIa analog (V158D/E296V/M298Q) in tissue factor-independent mechanisms*

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Summary

Factor (F)VIIa with tissue factor (TF) is a primary trigger of blood coagulation. The recombinant (r)FVIIa analog, NN1731 (V158D/E296V/M298Q) containing a thrombin/FIXa-mimicking catalytic domain, is ~30-fold more effective on activated platelets without TF, but ~1.2-fold with TF, than rFVIIa for FX activation. We have recently demonstrated the FVIIa/TF-dependent FVIII activation in the early coagulation phase. We assessed the action of NN1731 on FVIII activation. NN1731/TF increased FVIII activity ~2.9-fold within 30 seconds, followed by rapid inactivation, and was slightly more active than rFVIIa/TF. NN1731-catalysed activation, however, was enhanced ~6-fold at 5 minutes (min), and its peak level persisted for ~30 min. NN1731/TF proteolysed FVIII at Arg^{740}, Arg^{372}, and Arg^{336}, similar to rFVIIa/TF, but cleavage by NN1731 alone was much slower at Arg^{336} than at Arg^{740} and Arg^{372}. The K_m and V_{max} for NN1731/TF-catalysed activation were ~1.8-fold lower and ~2.3-fold greater than rFVIIa/TF. The K_m for NN1731 alone was ~1.3-fold lower than rFVIIa, whilst the V_{max} was ~7.9-fold greater, indicating that the efficiency of FVIII activation by NN1731 and NN1731/TF was ~11- and ~4-fold greater, respectively, than equivalent reactions with rFVIIa. In SPR-based assays, NN1731 bound to FVIII and the heavy chain (K_d; 0.62 and 1.9 nM) with ~1.4- and ~3.1-fold higher affinity than rFVIIa, and the A2 domain contributed to this increase. Von Willebrand factor moderated NN1731-catalysed activation more significantly than NN1731/TF. In conclusion, NN1731 was a greater potential than rFVIIa in up-regulating FVIII activity, and the TF-independent FVIII activation might represent a potential extra mode of its enhanced haemostatic effect.

Keywords

Factor VIII(a), factor VIIa-analog, activation, cleavage, tissue factor

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Introduction

Factor (F)VIII, a plasma protein deficient or defective in individuals with the severe congenital bleeding disorder, haemophilia A, functions as a cofactor in the tenase complex, responsible for phospholipid (PL)-dependent FXa generation by FIXa (1). FVIII is synthesised as a single chain molecule consisting of 2,332 amino acid residues, and is arranged into three domains, A1-A2-B-A3-C1-C2. FVIII is processed into a series of metal ion-dependent heterodimers by cleavage at the B-A3 junction, generating a heavy chain (HCh) comprising A1 and A2 domains together with partially proteolysed B domains linked to a light chain (LCh) consisting of A3, C1, and C2 domains. FVIII circulates in plasma as a complex with von Willebrand factor (VWF) (2). The catalytic efficiency of FVIII is markedly enhanced by conversion into an active form, FVIIIa, by limited proteolysis by thrombin (3). The enzyme proteolyses FVIII at Arg^{372} and Arg^{740} in the HCh, and produces 50-kDa A1 and 40-kDa A2 subunits. The 80-kDa LCh is cleaved at Arg^{1689} generating a 70-kDa ^{1690}A3C1C2. Proteolysis at Arg^{372} and Arg^{1689} is essential for generating FVIIIa cofactor activity (4). Cleavage at the former or latter site exposes a functional FIXa-interactive site within the A2 domain (5), or liberates FVIII from VWF (6). FVIIIa activity is down-regulated by several serine proteases including activated protein C (APC), following cleavage at Arg^{336} within the A1 subunit (3). This inactivation appears to reflect loss of a FX-interactive site, mediated by modified interaction with the A2 and an increased K_m of the truncated A1 for FX (7).

FVIIa is a serine protease responsible for initiating blood coagulation in normal haemostasis. The central role of FVIIa is the activation of FX (and FIX) (8). Following injury to the blood vessel wall, FVIIa forms a complex with tissue factor (TF) exposed to circulating blood, resulting in initiation of haemostasis through FX activation and the generation of trace amounts of thrombin (9). This thrombin activates FV and FVIII and promotes platelet activation. Following these "priming" reactions, thrombin generation is accelerated through propagation of tenase and prothrombinase enzymes on negatively-charged PL membrane exposed on platelet surfaces (10).

In our recent study (11), we demonstrated that FVIIa/TF activated FVIII and then inactivated FVIII by limited proteolysis at Arg^{372} (and Arg^{740}) and Arg^{336} in the HCh, respectively. In contrast, this enzyme did not proteolyse at Arg^{1689} in the LCh. Due to the inhibition of LCh cleavage, the FVIII activation by FVIIa/TF appeared to result in ~10% of peak level relative to full activation of FVIII by thrombin. This was supported by an earlier report (12) by mutational analysis using R1689A FVIII mutant. Intriguingly, FVIIa/TF-catalysed activation of FVIII was more rapid than that by thrombin, however. In addition, FVIIa/TF could activate FVIII to some extent even in the presence of VWF. We proposed, therefore, that as well as interaction with FIX and/or FX, FVIII (complexed with VWF) was initially activated by FVIIa/TF on the surface of injured vessel walls, contributing to the initiation of haemostasis by enhancing the assembly of intrinsic tenase and by leading to the rapid local generation of thrombin in the early phases of coagulation (11).

Recombinant (r)FVIIa products have been used for the treatment of bleeding in haemophilia A patients with inhibitors, acquired haemophilia, and congenital FVII deficiency. The action of rFVIIa is mediated by TF-dependent and/or TF-independent mechanisms (13, 14). At pharmacological dosages (~100 µg/kg), rFVIIa binds to locally activated platelets independent of TF following vascular damage. FX is activated directly on the platelet surfaces, resulting in enhanced localised thrombin generation and the formation of stable fibrin clots (15). The initial platelet activation is mediated by FVIIa binding to TF exposed after tissue injury, and consequently the action of therapeutic rFVIIa is restricted to injured sites. More recently, a FVIIa analog NN1731, a variant of rFVIIa with three amino acid substitutions (V158D/E296V/M298Q) containing a thrombin/FIXa-mimicking catalytic domain, has been suggested to be more effective than rFVIIa products (16). Similar to rFVIIa, NN1731 binds to TF, generating similar amounts of TF-dependent FXa. In addition, NN1731 binds to activated platelets, but its enzymatic activity is much greater than rFVIIa (17). In a cell-based model of FX activation *in vitro*, NN1731 appeared to be ~30-fold more potent than rFVIIa on activated platelets in the absence of TF, and ~1.2-fold greater in the presence of TF-expressing monocytes (18). This property of NN1731 has been confirmed in animal (19) and *in vitro* human models (20). A clinical development program for NN1731 is ongoing for haemophilia A patients with inhibitors.

In the present study, we compared NN1731 with rFVIIa in mechanisms of FVIII activation. We demonstrated that NN1731 had a greater potential than rFVIIa as an activator for the up-regulation of FVIII activity. In addition, the enhanced catalytic activity of NN1731 was most evident in the absence of TF, and appeared to be less related to binding affinity. TF-independent FVIII activation might represent a potential extra mode of its enhanced haemostatic effect.

Materials and methods

Reagents

Purified rFVIII preparations were a generous gift from Bayer Corp. Japan (Osaka, Japan). rFVIIa and its analog (NN1731) were kindly provided by Dr. Mirella Ezban (Novo Nordisk, Bagsværd, Denmark). FVIIIa, LCh (^{1649}A3C1C2 and ^{1690}A3C1C2), HCh (A1-A2-B), A1, and A2 subunits of FVIII were isolated and purified from rFVIII (21). The rA3 and rC2 domains of FVIII were expressed and purified as previously described (22, 23). VWF was isolated and purified from FVIII/VWF concentrates using gel filtration and immune-beads coated with immobilised FVIII mAb (24). SDS-PAGE of the isolated subunits followed by staining with GelCode Blue Reagent (Pierce, Rockford, IL, USA) showed >95% purity. Protein concentrations were determined by the Bradford's method. The mAbC5 (25), recognising the acidic region of the A1 domain, was a generous gift from Dr. Carol Fulcher (Scripps Clinic, La Jolla, CA, USA). Human FIXa and FX (Hematologic Technologies, Essex Junction, VT, USA), FXa (Enzyme Research Laboratories, South Bend, IN, USA), FVIIa-specific inhibitor peptide E-76 (Bachem, Torrance, CA, USA), chromogenic Xa substrate S-2222 (Chromogenix, Milano, Italy), recombinant lipidated TF (Innovin®; Dade Behring, Marburg, Germany), and Glu-Gly-Arg-chloromethylketone (EGR-ck; Calbiochem, San Diego, CA, USA) were purchased from the indicated vendors. FVIII-deficient plasmas were purchased from George King Biomedical (Overland Park, KS, USA). All other coagulation factors in these plasmas were within normal activity levels. PL vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine (Sigma, St Louis, MO, USA) were prepared using *N*-octylglucoside (26).

Active-site modified EGR-NN1731

NN1731 was inactivated by the addition of a 10-fold molar excess of EGR-ck in 50 mM HEPES, pH 7.2, and 0.1 M NaCl and incubation overnight at 4°C. Unbound EGR-ck was removed by extensive dialysis at 4°C in 20 mM HEPES, pH 7.2, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.01% Tween 20 (HBS-buffer) (11). Inactivation of NN1731 was considered complete when residual FVIIa activity was <0.2% as measured in FVIIa-specific assay.

Clotting assay

FVIII activity was measured in a one-stage clotting assay using FVIII-deficient plasma. All reactions were performed at 37°C. FVIII products were incubated in HBS-buffer containing 0.1% bovine serum albumin (BSA) plus the indicated concentrations of NN1731, PL and TF. Samples were removed from mixtures at the indicated times, and FVIIa/TF reaction was immediately terminated by the addition of E-76 (2.5 µM) and 2,500-fold dilution. The presence of FVIIa/TF and E-76 in the diluted sample did not affect FVIII activity (<5%) in the coagulation assay.

FXa generation assay

The rate of conversion of FX to FXa was monitored in a purified system. FXa was generated at 37°C in HBS-buffer containing 0.1% BSA. Various concentrations of FVIII and PL (80 μM) were incubated with FVIIa products (30 nM) without TF or with FVIIa products (0.1 nM) with TF (1 nM). The FVIIa reaction was terminated after 10 seconds (sec) by the addition of E-76 (2.5 μM). FXa generation was initiated by the addition of 2 nM FIXa and 200 nM FX. Aliquots were removed at the indicated time to assess initial rates of product formation, and added to tubes containing EDTA (100 mM) to stop the reaction. Rates of FXa generation were determined at 405 nm using a microtiter plate reader after the addition of chromogenic substrate, S-2222 (0.46 mM).

SDS-PAGE and Western blotting for FVIII cleavage

NN1731 (30 nM) in the presence or absence of 1 nM TF were added to 30 nM FVIII with 80 μM PL in HBS-buffer at 37°C. Aliquots were removed at the indicated times and the reactions were immediately terminated by adding SDS and boiling for 3 minutes (min). SDS-PAGE and Western blotting was performed using 8% gels at 150 V for 1 hour. Protein bands were probed using the indicated anti-A1 mAbC5 followed by goat anti-mouse IgG using an enhanced Chemifluorescence Western Blotting Kit (GE Healthcare, Little Chalfont, UK). Densitometric scans were quantitated using FLA3000 (Fuji Film, Tokyo, Japan)

Surface plasmon resonance (SPR)-based assay

The kinetics of FVIII and EGR-NN1731 interaction were determined by SPR-based assay using a BIAcore X instrument (Biacore AB, Uppsala, Sweden) (11). The reactions were run at 37°C. EGR-NN1731 was covalently coupled (0.4 ng/mm^2) to the CM5 chip. Association of the ligand was monitored at a flow rate of 30 μl/min for 2 min, and dissociation of bound ligand was recorded over a 2 min-period by replacing with buffer alone. Nonspecific binding, corresponding to ligand binding to the uncoated chip, was subtracted from the signal. Rate constants for association (k_{asso}) and dissociation (k_{diss}) were determined using the commercial evaluation software (Biacore AB).

Data analysis

All experiments were performed at least four separate times, and means ± SD are shown. Nonlinear least squares regression analyses were performed using Kaleida Graph (Synergy Software, Reading, PA, USA). Kinetics parameters were determined in FXa generation assays. The K_m and V_{max} values were calculated by fitting the data using nonlinear least squares regression analysis in the Michaelis-Menten equation.

Results

FVIII activation by NN1731

Firstly, we examined whether NN1731 as well rFVIIa contributed to the activation and inactivation of FVIII. FVIII (30 nM) was incubated with the FVIIa product (30 nM) together with PL (80 μM) in the presence or absence of TF (1 nM), and FVIII activity was measured in a one-stage clotting assay. To eliminate the influence of FVIIa and TF/PL in this assay, 2,500-fold dilutions of reactant mixtures were utilised. The lowest level of FVIII activity detected in these assays was ~0.01 nM, and under these conditions, therefore, FVIII was used at ~30-fold higher than physiological concentrations. In addition, the pharmacological dose of rFVIIa is ~100 μg/kg (~25 nM in plasma), and the rFVIIa reactions *in vitro* in the absence of TF were very weak. As a consequence, FVIIa was utilised at 30 nM in these experiments. Time-course reactions of FVIII activation by NN1731 (panel a) and rFVIIa (panel b) are shown in ▶Figure 1A. In the presence of TF (1 nM; closed triangles), NN1731 enhanced FVIII activity very rapidly (<30 sec) by ~2.9-fold over the base level, and appeared to be slightly more potent than rFVIIa (~2.6-fold increase). This elevated FVIII activity rapidly decreased and fell below initial levels after ~10 min. In contrast in the absence of TF (open circles in ▶Fig. 1A), NN1731 promoted FVIII activity by 5~6-fold in a time-dependent manner over 5 min, and the plateau level remained constant for ~30 min after reaction. Furthermore, this peak level was ~2-fold greater than that in the presence of TF (1 nM). The increase in FVIII activity mediated by NN1731 was PL-dose dependent. The reaction was saturable at a concentration of 80 μM and less evident in the absence of PL (data not shown). Although rFVIIa alone increased FVIII activity very slowly, the peak activity was shown to be limited to ~1.7-fold of the initial level even after 30-min reaction. These results demonstrated that TF influenced NN1731 and rFVIIa reactions with FVIII in a similar manner, but NN1731 mediated significant activation of FVIII (as well as FX) in the presence of PL surface even in the absence of TF.

The role of TF in the pattern of FVIII activation by NN1731

Our results indicated that the mechanisms of NN1731- and rFVIIa-catalysed activation of FVIII were similar in the presence of TF (1 nM), but were significantly different in the absence of TF. Further experiments were designed, therefore, to compare FVIII activation by NN1731 and rFVIIa in the presence of various concentrations of TF. In the absence of TF, NN1731 induced an incremental increase in FVIII activity up to ~6-fold (▶Fig. 1A, panel a). The addition of TF depressed the plateau peak level of FVIII activity at >5 min in a dose-dependent manner, and FVIII activity decreased to below the initial level in the presence of TF (>1 nM). In contrast, FVIII activity in early-reaction phases with NN1731 (<30 sec) was increased dose-dependently by the addition of TF. In particular, FVIII activity with TF (>0.5 nM) reached maximum peak

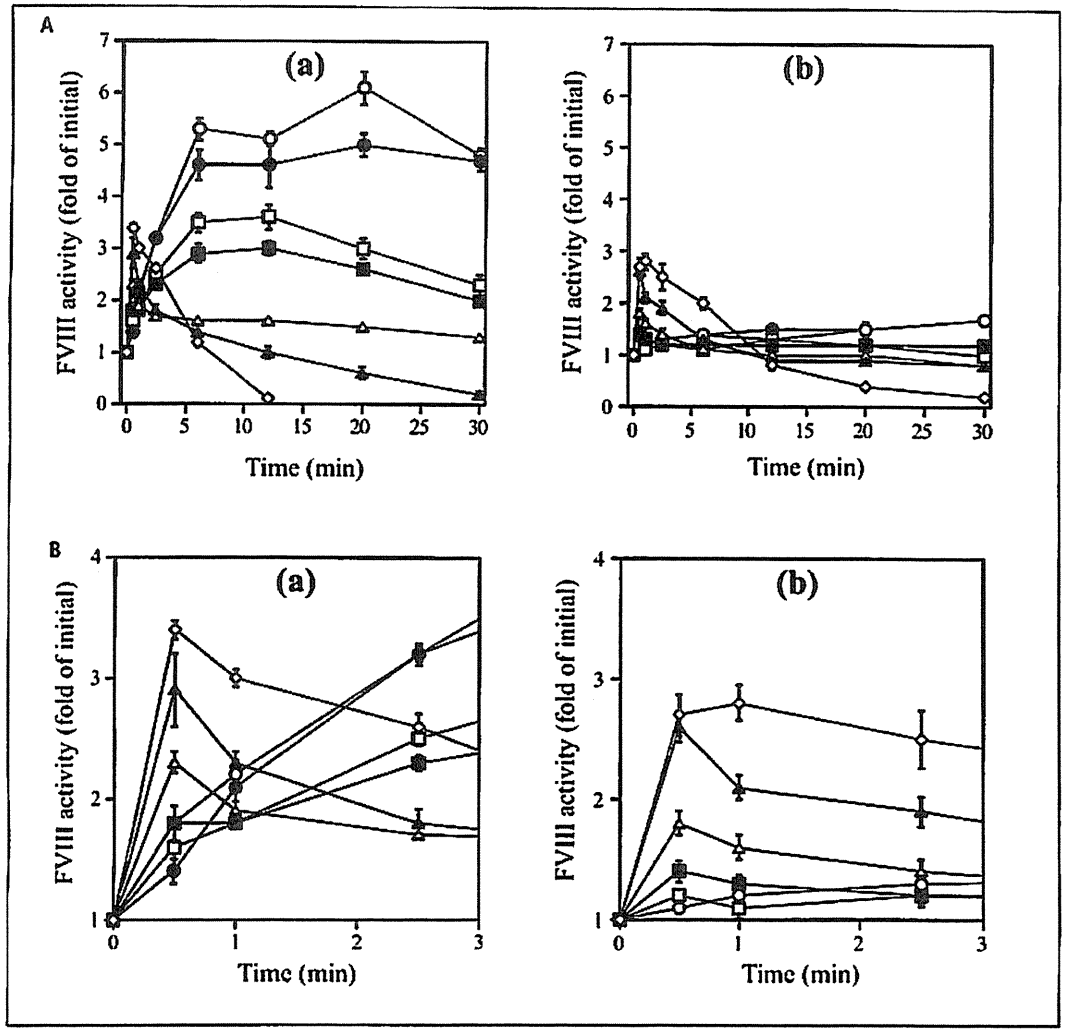

Figure 1: Activation of FVIII following reaction with NN1731 or rFVIIa in the presence of TF. A) FVIII (30 nM) was incubated with 30 nM NN1731 (panel a) or rFVIIa (panel b) with PL (80 µM) and various concentrations of TF (0 nM, ○; 0.01 nM, ●; 0.1 nM, □; 0.2 nM, ■; 0.5 nM, Δ; 1.0 nM, ▲; 2.0 nM, ◊) for the indicated times (0–30 min), after which the reaction was terminated by E-76 peptide. Each sample was tested immediately for FVIIIa activity in a one-stage clotting assay. B) Enlarged time course of NN1731 (panel a) or rFVIIa (panel b) reaction for 0–2.5 min in (A), respectively. The initial activity of FVIII was ~25 units/ml. Experiments were performed at least four separate times and means ± SD are shown.

levels during a 30 sec-reaction period (▶Fig. 1B, panel a). Conversely, rFVIIa in the absence of TF elevated FVIII activity very slowly (by ~1.7-fold), whilst rFVIIa with TF promoted FVIII activity rapidly within 30 sec in a dose-dependent manner (▶Fig. 1A, panel b). In the presence of TF (>1 nM), the pattern of rFVIIa-catalysed reaction was similar to that for NN1731 (▶Fig. 1B, panel b). These findings demonstrated that the biphasic reactions for activation and inactivation of FVIII appeared to be dramatically dependent of the presence of TF in both NN1731 and rFVIIa. Changes were more predominant for NN1731 in the presence of small amounts of TF (<0.2 nM).

FVIII proteolysis by NN1731

FVIII is activated very rapidly by rFVIIa/TF by proteolysis at Arg^{372} and Arg^{740} in the HCh, and subsequently inactivated following proteolysis at Arg^{336}. No cleavage of the LCh is evident in early-timed reactions (<30 min) (11). In the present study we noted that FVIII reactions mediated by NN1731 and rFVIIa in the presence of TF were different from those in its absence, and we further investigated, therefore, NN1731-catalysed proteolytic cleavage of FVIII using SDS-PAGE. Proteolysed fragments were visualised by Western blotting using anti-A1 mAbC5 recognising the A1 acidic region (▶Fig. 2A, B). Disappearance and/or appearance of the A1-A2-B, $A1^{1-372}$, and $A1^{337-372}$-A2, reflecting cleavage at Arg^{740}, Arg^{372}, and Arg^{336}, respectively, was quantitated by band densitometry (▶Fig. 2C). Cleavage sites of FVIII fragments derived from NN1731 proteolysis (with/without TF) were identified using automated NH_2-terminal sequence analysis. These cleavage sites were confirmed to be identical to those derived from rFVII/TF proteolysis (data not shown). Although HPLC-gel filtration was used to fractionate intact FVIII, $A1^{1-372}$ fragments remained evident even in the absence of FVIIa, indicating a possible high sensitivity of mAbC5. In the presence of TF (▶Fig. 2A), both NN1731 (panel a) and rFVIIa (panel b) rapidly (<0.5 min) proteolysed the HCh (A1-A2-B) into A1-A2 fragments by cleavage at Arg^{740}, and this was followed by sequential generation of $A1^{1-372}$ fragments by cleavage at Arg^{372}. Subsequently, $A1^{337-372}$-A2 fragments mediated

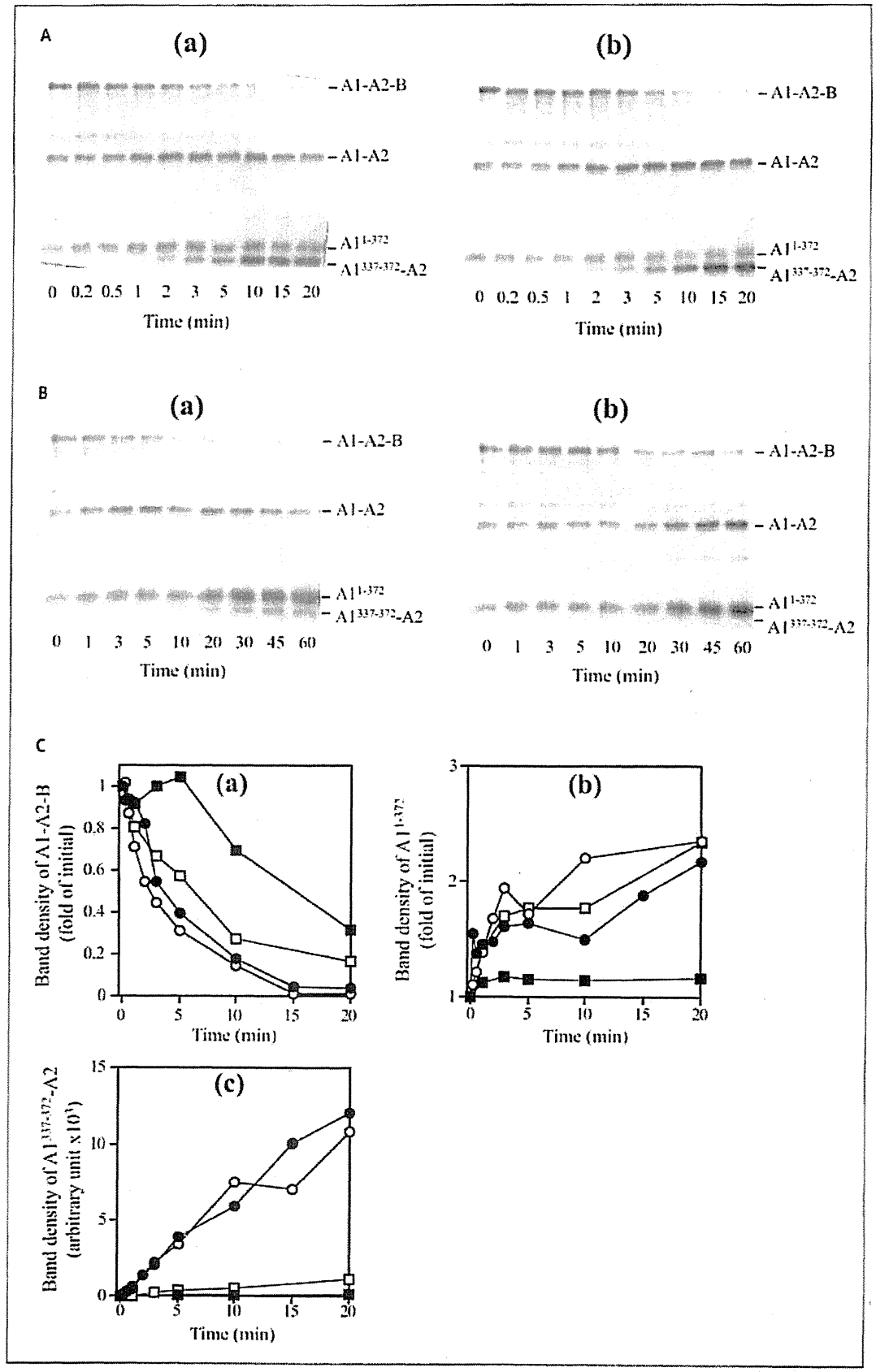

Figure 2: Comparison with FVIII cleavage by NN1731 and rFVIIa. FVIII (30 nM) was incubated with 30 nM NN1731 (panel a) or rFVIIa (panel b) in the presence (A) or absence (B) of TF (1 nM) with PL (80 μM) for the indicated times (plus TF; 0–20 min, minus TF; 0–60 min). Samples were run on 8% gels followed by Western blotting using anti-A1 mAbC5. C) Data (reaction for 0–20 min) obtained by quantitative densitometry of the intact A1-A2-B (panel a), $A1^{1-372}$ (panel b), and $A1^{337-372}$-A2 (panel c) in (A) and (B). The symbols used are: NN1731/TF, ○; rFVIIa/TF, ●; NN1731, □; rFVIIa, ■.

by cleavage at Arg^{336} were visualised at ~1 min. These findings were consistent with rapid up- and down-regulation of FVIII, although cleavage by NN1731/TF was somewhat faster than that by rFVIIa/TF. In the absence of TF (▶ Fig. 2B), NN1731 (panel a) rapidly, and time-dependently proteolysed A1-A2-B into A1-A2 fragments by cleavage at Arg^{740}, followed by generation of $A1^{1-372}$ fragments by cleavage at Arg^{372}. Subsequently, cleavage at Arg^{336} led to the appearance of $A1^{337-372}$-A2 fragments, ~10 min after reaction with NN1731. These data were in keeping with the peak level of FVIII activity observed during FVIII activation. In contrast, cleavage at Arg^{740} and Arg^{372} was markedly slower with rFVIIa (panel b) than with NN1731, and cleavage at Arg^{336} appeared to be minimal within 60 min. These results were consistent with the differences in FVIII procoagulant activation mediated by NN1731 and rFVIIa in the absence of TF. Little cleavage of the LCh was observed in these experiments (data not shown), indicating that LCh reactions were not associated with FVIII activation by NN1731 or rFVIIa.

Kinetic analysis of FVIII activation by FVIIa products in FXa generation assays

To further investigate the functional mechanisms of NN1731 and rFVIIa with FVIII, kinetic parameters of FVIII activation by FVIIa products were determined in FXa generation assays. Various concentrations of FVIII and PL (80 μM) were incubated with FVIIa products (30 nM) without TF or with FVIIa products (0.1 nM) with TF (1 nM). Reactions were terminated at 10 sec, reflecting FVIII activation alone, by the addition of E-76. FXa generation was initiated by the addition of FIXa (2 nM) and FX (200 nM). Non-activated FVIII alone had little effect on FXa generation under these conditions (data not shown), and the rate of FXa generation was considered, therefore, to directly reflect the concentration of activated FVIII. Results are shown in ▶ Figure 3 and summarised in ▶ Table 1. In the presence of TF (▶ Fig. 3A), the K_m obtained for FVIII activation by NN1731 was ~1.8-fold lower than for rFVIIa (27.3 and 49.2 nM, respectively), and the V_{max} obtained for FVIII activation by NN1731 was ~2.3-fold greater (70.0 and 30.4 nM·min^{-1}, respectively). In the absence of TF, however (▶ Fig. 3B), although the K_m obtained by NN1731 was only ~1.3-fold lower than that seen with rFVIIa (50.5 and 68.1 nM, respectively), the V_{max} obtained by NN1731 was ~7.9-fold greater (92.5 and 11.7 nM·min^{-1}, respectively). Overall, in the absence of TF the catalytic efficiency of NN1731 for FVIII activation (V_{max}/K_m) was ~11-fold greater than that of rFVIIa, whilst in the presence of TF this measurement was ~4-fold greater for NN1731 than for rFVIIa. In this experiment, the concentrations of protease in the presence of TF were 300-fold lower than those in its absence, i.e. the apparent k_{cat} values of NN1731 and rFVIIa in the presence of TF were ~230- and ~780-fold higher, respectively, than those in its absence, and the affinities of NN1731 and rFVIIa for FVIII activation in the presence of TF were ~1.9- and ~1.4-fold higher than those in its absence.

Binding of FVIII(a) subunits to EGR-NN1731 in SPR-based assays

We have previously established a direct method for assessing interactions of FVIII with EGR-rFVIIa, in place of native rFVIIa, using SPR-based assays (11). Similar experiments were developed, there-

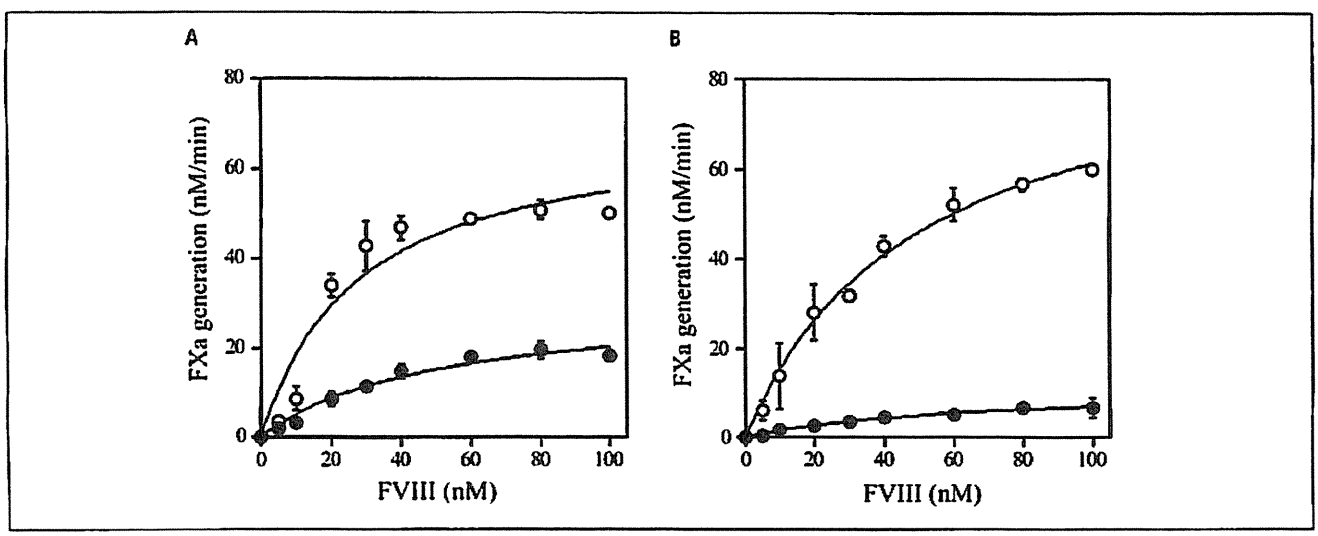

Figure 3: Kinetics analyses on FVIII activation caused by NN1731 and rFVIIa in FXa generation assay. Various concentrations of FVIII and PL (80 μM) were activated by 0.1 nM NN1731 (○) or rFVIIa (●) in the presence of TF (1 nM) (A) or activated by 30 nM NN1731 (○) or rFVIIa (●) in the absence of TF (B). The reaction was terminated at 10 sec by the addition of E-76. FXa generation was initiated by the addition of FIXa (2 nM) and FX (200 nM). Initial rates of FXa generation on FVIII activation by NN1731 and rFVIIa were plotted as a function of FVIII concentration and fitted to the Michaelis-Menten equation by non-linear least squares regression. Experiments were performed at least five separate times, and mean ± SEM values are shown.

fore, to study interactions of FVIII and EGR-NN1731. Representative curves for FVIII and EGR-NN1731 binding are shown in ▶Figure 4. Binding parameters are summarised in ▶Table 2. FVIII and its active form (FVIIIa) bound to NN1731 with ~1.4-fold higher affinity (K_d; 0.62 and 5 nM) than to rFVIIa. In the competitive binding assay using EGR-FVIIa, we confirmed that the EGR-FVIIa inhibited the FVIII and EGR-FVIIa binding in similar dose-dependent manner (data not shown), supportive of validity of this assay. The HCh bound to NN1731 (K_d; 1.9 nM) with ~3.1-fold higher than to rFVIIa. These differences were more predominant than those with the LCh (by ~2.2-fold). In particular, the A2 fragment bound to NN1731 with ~2.1-fold higher affinity (K_d; 12.5 and 26.4 nM), indicating a likely significant contribution of this higher affinity for reactions of NN1731. No binding of the A1 domain was evident in these experiments. Thrombin-cleaved LCh ($^{1e/90}$A3C1C2) bound to NN1731 with a similar affinity to LCh, suggesting that the acidic region of the A3 domain was not an essential component of the NN1731-interactive site. The A3 and C2 fragments of LCh bound to NN1731 with a similar and less affinity than to rFVIIa. Overall, the findings suggested that the binding affinity of NN1731 for FVIII was somewhat greater than that of rFVIIa, and supported the results obtained in FXa generation assays, although the K_m values obtained in the enzyme assays were much greater than the K_d values in this binding assay. In addition, the A2 domain appeared to play a significant role in interactions with NN1731 as well as with rFVIIa.

Effect of VWF on FVIII activation by NN1731

Our previous study showed that the presence of VWF decreased maximum binding of FVIII to rFVIIa by ~50% and only modestly counteracted rFVIIa-catalysed FVIII activation, suggesting that VWF partially modulated FVIIa/TF-mediated activation of FVIII by direct competition with FVIII molecule (11). We have compared, therefore, the effects of VWF on NN1731– and rFVIIa-catalysed activation of FVIII using one-stage clotting assays. FVIII (30 nM) was preincubated with various concentrations of VWF prior to reaction with 30 nM NN1731 (panel a) or rFVIIa (panel b) in the presence of PL (80 μM) and TF (1 nM). In the presence of TF (▶Fig. 5A), VWF similarly and modestly moderated the spiked activation of FVIII by NN1731 and rFVIIa, and the subsequent inactivation reactions were also depressed in dose-dependent manners. In these experiments, VWF (20 μg/ml) inhibited FVIII activation by NN1731 and rFVIIa by only ~20%. In contrast, in the absence of TF (▶Fig. 5B), VWF inhibited FVIII activation by both products more significantly. VWF at physiological concentrations (10 μg/ml) decreased the peak activity of FVIII activation by NN1731 and rFVIIa by ~90% and ~85%, respectively. These findings were supported by data obtained from FVIII proteolysis by the FVIIa products in Western blotting. In the presence of TF, cleavage at Arg^{336} by both NN1731 and rFVIIa was inhibited by VWF dose-dependently, and this inhibition was more prominent than that at Arg^{372} and Arg^{740}. In the absence of TF, inhibition of NN1731-mediated cleavage at Arg^{372} and Arg^{740} appeared to be more significant, although inhibition of rFVIIa-induced cleavage at these sites was poorly observed (data not shown). The results indicated, therefore, that VWF inhibited NN1731-catalysed proteolysis of FVIII more potently in the absence of TF than in its presence.

Discussion

NN1731 is an analog of rFVIIa, in which three amino acid residues are substituted (V158D/E296V/M298Q), containing a thrombin/

Table 1: Kinetic parameters of FVIII activation by NN1731 and rFVIIa in FXa generation assays. Reactions were performed as described in *Methods*. Parameters were calculated by non-linear regression analysis using the Michaelis-Menten equation. Experiments were performed at least five separate times, and mean ± SEM values are shown.

	K_m	V_{max}	V_{max}/K_m
	nM	nM·min^{-1}	min^{-1}
NN1731/TF	27.3 ± 10.9	70.0 ± 10.3	2.56
rFVIIa/TF	49.2 13.5	30.4 ± 3.9	0.62
ratio	*(1.8–1)*	*(2.3)*	*(4.1)*
NN1731	50.5 ± 6.9	92.5 ± 6.0	1.83
rFVIIa	68.1 ± 17.4	11.7 ± 1.5	0.17
ratio	*(1.3–1)*	*(7.9)*	*(10.8)*

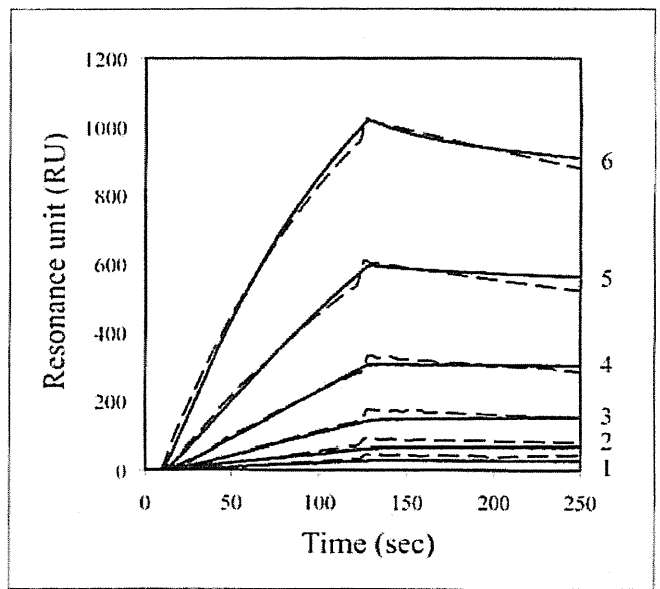

Figure 4: Direct binding of FVIII to EGR-NN1731. Various concentrations of the FVIII were injected to the EGR-FVIIa (0.4 ng/mm^2) immobilised onto the sensor chip at a flow rate of 30 μl/min for 2 min, followed by the change of running buffer for 2 min. The solid and dashed lines 1–6 show representative response curves for the different concentrations of FVIII (0.315, 0.625, 1.25, 2.5, 5, 10 nM, respectively) and fitting curves.

Table 2: Binding parameters between FVIII(a) subunit and EGR-FVIIa interaction in a SPR-based assay. Reactions were performed as described in *Methods*. Parameters were calculated by non-linear regression analysis using the evaluation software provided by Biacore AB. Experiments were performed at least five separate times, and mean ± SEM values are shown. The K_d values were calculated as k_{diss}/k_{ass}. *: Not determined.

Ligands	NN1731			rFVIIa	
	k_{ass}	k_{diss}	K_d	$K_d^{(11)}$	$K_d^{rFVIIa}/K_d^{NN1731}$
	x10^4 $M^{-1}s^{-1}$	x10^{-3} s^{-1}	nM	nM	-fold
FVIII	297 ± 9	1.84 ± 0.03	0.62	0.82	1.3
FVIIIa	200 ± 11	9.93 ± 0.66	5.0	7.4	1.5
HCh	47.1 ± 1.2	0.89 ± 0.05	1.9	5.9	3.1
^{1649}A3C1C2	44.7 ± 1.0	3.82 ± 0.05	8.6	18.9	2.2
^{1690}A3C1C2	60.2 ± 4.3	7.10 ± 0.39	11.8	15.1	1.3
A1	n.d.*	n.d.*	-	-	-
A2	9.75 ± 0.60	1.22 ± 0.06	12.5	26.4	2.1
A3	4.35 ± 0.49	8.33 ± 0.28	191	273	1.4
C2	0.87 ± 0.03	6.32 ± 0.09	726	376	0.52

FIXa-mimicking catalytic domain. It is a more potent FX activator than rFVIIa in the presence of TF, and in particular activates FX strongly even in the absence of TF (17, 18). Recently we have further developed a previous study by Warren et al. that showed that FVIIa/TF catalysed proteolyses of FVIII (27), and suggested that FVIIa/TF activated not only FX and FIX, but also FVIII in physiological mechanisms of blood coagulation (11). In the present study, we compared the activation of FVIII mediated by rFVIIa and NN1731.

The rapid reaction of FVIII by NN1731 in the presence of TF appeared to be somewhat greater than that by rFVIIa. In its absence, however, NN1731 markedly increased FVIII activity, but rFVIIa did not significantly proteolyse the HCh. The peak level of NN1731-mediated FVIII activity was depressed by TF dose-dependently, although interestingly, even trace amounts of TF (<~0.2 nM) markedly shortened the time to peak of FVIII activation by NN1731. The peak activity mediated by NN1731/TF was lower by ~2-fold than that by NN1731 alone. FVIII inactivation associated with cleavage at Arg^{336} was evident only in the presence of TF, and TF dramatically altered not only cleavage velocity, but also the reaction pattern of NN1731-mediated FVIII activation and inactivation. Overall, we demonstrated that in the absence of TF, NN1731 likely revealed thrombin-like protease characteristics rather than those of native FVIIa on FVIII, whilst, in its presence, NN1731 behaved as more FVIIa-like than thrombin-like in these mixtures.

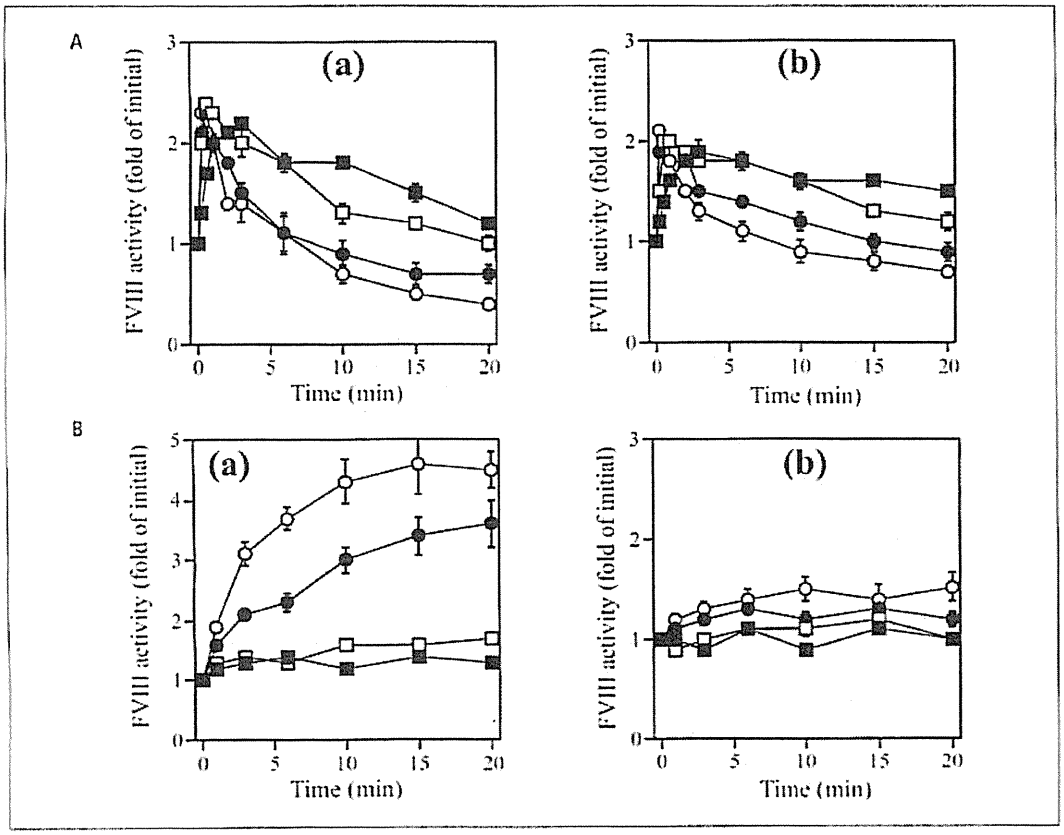

Figure 5: Effect of VWF on NN1731-catalysed reaction of FVIII. FVIII activation: FVIII (30 nM) was preincubated with various concentrations of VWF (0 μg/ml, ○; 5 μg/ml, ●; 10 μg/ml, ⊥; 20 μg/ml, ■) prior to reaction with 30 nM NN1731 (panel a) or rFVIIa (panel b) in the presence (A) or absence (B) of TF (1 nM) with PL (80 μM). Each sample was tested immediately for FVIIIa activity in a one-stage clotting assay. The initial activity of FVIII was ~25 units/ml. Experiments were performed at least four separate times and means ± SD are shown.

The V_{max} of NN1731-catalysed FVIII activation was significantly greater than that of rFVIIa in the absence of TF, but this was marginally greater in its presence. While, the K_m of FVIII and NN1731 interaction was marginally greater than that of rFVIIa, independently of TF. These findings supported that this potential of NN1731 without TF appeared likely to attribute to the catalytic activity of NN1731 rather than the binding affinity. Although SPR-based assays showed that NN1731 bound to FVIII slightly tightly compared to rFVIIa, the binding affinity observed in this method (K_d) was ~80-fold greater than that seen in FXa generation (K_m). This discrepancy may have been due to conformational changes in the presence of PL. An earlier report described that the increase for K_m on FX (or FIX) activation by FVIIa was dependent on the presence of PL (8). Alternatively, changes in binding affinity of EGR-NN1731 could have contributed to the findings. Some types of active-site-blocked FVIIa with soluble TF had been reported to possess ~5-fold higher affinity than native FVIIa (28), suggesting that the affinities of FVIII and EGR-FVIIa may be miscalculated.

Similar to rFVIIa/TF, NN1731/TF-mediated FVIII activation was partially moderated by the presence of VWF at the physiological concentration. Distinct from PL-dependent serine proteases that were regulated by VWF, however, rFVIIa and NN1731 in the presence of TF could activate FVIII complexed with VWF to a significant extent, although the FVII/TF-catalysed FVIII activation in the absence of VWF was reached at ~10% level of full activation of FVIII by thrombin due to the inhibition of LCh cleavage. From this finding, TF might compete with VWF for binding to FVIII, resulting in modest activation of FVIII by rFVIIa (and NN1731) with TF even in the presence of VWF. This potential interaction is currently under investigation. However, VWF at physiological concentrations significantly inhibited (by ~90%) rFVIIa- and NN1731-mediated FVIII activation in the absence of TF, might suggesting that under the conditions without exposed TF, NN1731 (and rFVIIa)-mediated FVIII activation would hardly occur, except for the presence of anti-FVIII inhibitors that prevent the FVIII-VWF interaction (as described below).

It is difficult to determine precisely whether FVIIIa was generated from FVIII by thrombin or FVIIa/TF in these complex mixtures, and our suggestion that FVIIa/TF activates FVIII as well as FX (and FIX) in the initiation phase of coagulation remains to be confirmed in circulating blood. Nevertheless, two of the current findings especially indicated that this reaction could be physiologically relevant. Firstly, SPR-based assays using high flow rates (30 µl/min) revealed high affinities for EGR-FVIIa products with FVIII (K_d; 0.6~0.8 nM), suggesting that FVIII at physiological concentrations (~1 nM) could interact with FVIIa under flow conditions. Although the binding affinities obtained using active-site modified FVIIa may be overestimated, the K_d value of EGR-FVIIa for FVIII was similar to that of active-site blocked FVIIa for soluble TF (28). Secondly, FVIII activation mediated by FVIIa/TF was more rapid (<30 sec) than that induced by thrombin (11). *In vivo*, the anti-coagulant protein tissue factor pathway inhibitor (TFPI) circulates in plasma. TFPI is complexed with generated FXa and abrogates the procoagulant activity of FVIIa/TF. Hence, significant inhibition of FVIIa/TF activity by TFPI/FXa complex was observed after 1 min *in vitro* (29). In a capillary model under flow conditions, adding TFPI completely blocked FXa generation induced by FVIIa/TF (30). In the presence of FVIII and FIX, however, FXa generation was observed at appreciable rates even at the same TFPI concentration. Taken together, FVIIa/TF reaction with FVIII under flow conditions could be presumed even in the presence of TFPI.

The clinical use of bypassing agents for haemophiliacs with inhibitor is well described (31). Furthermore, the presence of trace amounts of FVIII appears likely to govern the bleeding phenotype of haemophiliacs in the presence or absence of inhibitor (32). A number of inhibitory mechanisms of anti-FVIII inhibitors have been identified. Some inhibitors moderate FVIII activation by thrombin and FXa, and others limit FVIII-VWF interaction, FVIII-PL interaction, and FVIII-FIXa interaction (33). We have recently reported that FVIIa/TF-catalysed FVIII activation was not significantly affected by the various types of inhibitor (34). Thus,

What is known about this topic?

- The central role of factor (F)VIIa, a serine protease responsible for initiating blood coagulation in normal haemostasis, is the activation of FX and FIX.
- We have recently reported that FVIIa/tissue factor (TF) activates FVIII very rapidly by proteolysis at Arg^{372} and Arg^{740}, and it activates FVIII to some extent even in the presence of von Willebrand factor (VWF).
- As well as interaction with FIX and/or FX, FVIII (with VWF) is initially activated by FVIIa/TF on the injured vessel surface, may contributing to the initiation of haemostasis by enhancing the intrinsic tenase assembly and by leading to the rapid local generation of thrombin in the early phases of coagulation.
- A rFVIIa-analog, NN1731 (V158D/E296V/M298Q) containing a thrombin/FIXa-mimicking catalytic domain, is ~30-fold more effective on activated platelets without TF, but ~1.2-fold with TF, for FX activation, compared to rFVIIa. The action of NN1731 on FVIII activation remains unknown.

What does this paper add?

- In the presence of TF, NN1731 rapidly activated FVIII (~2.9-fold), followed by inactivation, and was slightly more active than rFVIIa. In its absence, NN1731-catalysed activation was enhanced ~6-fold at 5 min, and its peak level persisted for ~30 min.
- Both proteolysed the heavy chain at identical sites, independently of TF, but the deavage by NN1731 alone was much slower at Arg^{336} than at Arg^{740} and Arg^{372}.
- The catalytic efficiency of NN1731-catalysed FVIII activation without and with TF was ~11- and ~4-fold greater, respectively, than equivalent reactions with rFVIIa.
- VWF moderated NN1731-catalysed activation more significantly than NN1731/TF.
- NN1731 is greater potential than rFVIIa in up-regulating FVIII activity in the absence of TF, and the TF-independent FVIII activation may represent a potential extra mode of its enhanced haemostatic effect.

the ability of NN1731 to activate FX, FIX and FVIII even in the absence of TF could improve the therapeutic options for bleeding in haemophiliacs with inhibitor. The co-existence of FVIII and NN1731 in circulating blood could elicit improved responses in haemophiliacs with inhibitor. In particular, TF-independent NN1731 action to FVIII molecule might be very useful under the conditions of presence of anti-FVIII type I inhibitors, having the property of inhibition of FVIII-VWF association in circulating plasma (33).

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Conflict of interest

K.N. has received a grant from Bayer Hemophilia Award 2009.

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Effects of anti-factor VIII inhibitor antibodies on factor VIIa/tissue factor-catalysed activation and inactivation of factor VIII

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Summary

Factor (F)VIIa/tissue factor (TF) rapidly activates FVIII activity by proteolysis at Arg^{372} and Arg^{740}, and subsequently inactivates FVIIIa activity by proteolysis at Arg^{336}, although this activation is weaker than that by thrombin. The effects of anti-FVIII inhibitor antibodies on these reactions remain unknown, however. In this study, 13 of anti-FVIII inhibitor antibodies recognising the A2 or C2 domain were prepared. None of them, irrespective of epitope specificity, significantly affected FVIIa/TF-catalysed FVIII activation in one-stage clotting assays. Anti-A2 and anti-C2 type 2 antibodies had little effect on the inactivation phase. Anti-C2 type 1 antibodies, however, modulated inactivation by 40–60% of that seen with control IgG, suggesting that the activity of FVIIIa generated by FVIIa/TF persisted in the presence of this specific type of inhibitor. SDS-PAGE analysis demonstrated that all antibodies had little effect on FVIIa/TF-catalyzed proteolysis at Arg^{372} and Arg^{740}. Anti-C2 type 1, however, significantly delayed cleavage at Arg^{336} in dose-dependent manners. Neither anti-A2 nor anti-C2 type 2 affected this reaction, and the findings were consistent with the results of the functional assays. In addition, anti-C2 monoclonal antibodies with type 1 and 2 demonstrated similar patterns of reaction as the anti-C2 polyclonal antibodies in FVIIa/TF-mediated FVIII mechanisms. We demonstrated that FVIIa/TF activated FVIII even in the presence of anti-FVIII antibodies, but inactivation patterns appeared to depend on inhibitor type. It could be important to determine the characteristic of these inhibitor antibodies for prediction of their effects on FVIIa-related FVIII reactions, and the results could have significant therapeutic implications.

Keywords

FVIII, FVIII inhibitor antibodies, FVIIa/TF, activation/inactivation, cleavage

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Introduction

Factor (F)VIII, a plasma protein deficient or defective in individuals with the severe congenital bleeding disorder, haemophilia A, functions as a cofactor in the tenase complex, responsible for phospholipid (PL)-dependent conversion of FX to FXa by FIXa (1). FVIII is synthesised as a single chain molecule consisting of 2,332 amino acid residues (molecular weight [MW]: ~300 kDa), and is arranged into three domains, A1-A2-B-A3-C1-C2, based on amino acid homology. FVIII is processed into a series of metal ion-dependent heterodimers by cleavage at B-A3 junction, generating a heavy chain (HCh) consisting of A1 and A2 domains together with heterogeneous fragments of proteolysed 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 into an active form, FVIIIa, by limited proteolysis by thrombin and FXa (3). Both enzymes proteolyse the FVIII HCh at Arg^{372} and Arg^{740}, and generate 50-kDa A1 and 40-kDa A2 subunits. The 80-kDa LCh is cleaved at Arg^{1689} producing a 70-kDa subunit. Proteolysis at Arg^{372} and Arg^{1689} is essential for generating FVIIIa cofactor activity (4). Cleavage at the latter site liberates FVIII from its carrier protein, von Willebrand factor (VWF) (5). FVIIIa activity is down-regulated by serine proteases including activated protein C (APC), following cleavage at Arg^{336} (3). This inactivation appears to reflect loss of a FX-interactive site, mediated by a modified interaction with the A2 subunit and an increased K_m of the truncated A1 for the substrate FX (6).

FVIIa is a potent serine protease responsible for initiating and propagating the blood coagulation cascade in normal haemostasis (7). Following injury to the blood vessel wall, tissue factor (TF) exposed to circulating blood, forms a complex with FVIIa, resulting in the initiation of haemostasis by the activation of FX (and FIX)

and the generation of minimal amounts of thrombin (8). These trace amounts of thrombin dissociate FVIII from VWF and promote platelet activation. Following these "priming" reactions, thrombin generation is accelerated by the propagation of tenase and prothrombinase enzymes on PL micelles exposed on platelet surfaces (9). In addition, a TF-independent cell-based FX activation mechanism has been identified involving direct binding to platelet membranes (10), and these concepts in particular have been applied to haemostatic therapy in haemophilia patients with inhibitors.

FVIII inhibitors develop as alloantibodies (alloAbs) in 20–30% of multi-transfused patients with haemophilia A and also arise as autoantibodies (autoAbs) in previously normal individuals resulting in acquired haemophilia A and severe bleeding tendency (11). The decrease or disappearance of FVIII(a) activity in the presence of inhibitors is associated with impairment of FVIII(a) cofactor function mediated by binding to functionally essential regions in the FVIII molecule. FVIII inhibitors can either inhibit FVIII completely or incompletely at saturating concentrations, corresponding to a classification of type 1 or type 2, respectively (12). Inhibitory epitopes have been localised to one or more of the A2, A3-C1, and C2 domains (13–15). In general, anti-C2 inhibitor antibodies with type 1 pattern prevent FVIII binding to PL, VWF, and FXa, whilst those classified as type 2 prevent FVIII binding to thrombin and FXa (16–18). On the other hand, anti-A2 antibodies prevent FVIII interaction with FIXa and thrombin (19, 20), and anti-A3-C1 antibodies prevent FVIII(a) binding to FIXa (15).

We have recently reported that the FVIIa/TF complex rapidly activated FVIII by limited proteolysis at Arg^{372} and Arg^{740} in the HCh (21). FVIIa/TF appeared to promote a "priming" mechanism to generate small amounts of FVIIIa in the early initiation phases of coagulation, although the degree of this activation was lower than that mediated by thrombin, due to the rapid cleavage at Arg^{336} and the much slower cleavage at Arg^{1689}. The effects of anti-FVIII inhibitor antibodies on this mechanism remain unknown, however. In the present study, we have examined the FVIIa/TF-catalysed activation and inactivation of FVIII in the presence of anti-FVIII antibodies. We demonstrated that FVIIa/TF activated FVIII even in the presence of anti-FVIII antibodies, but inactivation patterns appeared to depend on their epitope specificity and types. These findings supported that it could be important to determine the characteristic of these FVIII inhibitor antibodies to predict their physiological effects on FVIIa-related FVIII reactions. In addition, the results could have significant therapeutic implications.

Materials and methods

Reagents

Purified recombinant FVIII and FVIIa preparations were generously provided by Bayer Corp. Japan (Osaka, Japan) and NovoNordisk (Bagsværd, Denmark), respectively. The A1, A2, HCh, LCh, and thrombin-cleaved LCh fragments were isolated and purified from recombinant FVIII as previously reported (6, 22). A cDNA coding the C2 domain sequence of human FVIII was constructed, transformed into *Pichia pastoris* cells and expressed in a yeast secretion system (23). The C2 protein was purified by ammonium sulfate fractionation and cation-exchange HPLC. The recombinant A3 domain of FVIII was expressed in *Escherichia coli* using the pET expression system (Novagen, Madison, WI, USA) (24), and the protein was purified using His-Select affinity cartridges. SDS-PAGE of the isolated subunits followed by staining with GelCode Blue Stain Reagent (Pierce, Rockford, IL, USA) showed >95% purity. The monoclonal antibody (mAb), C5 (25), recognising the C-terminal end of the A1 domain, was a generous gift from Dr. Carol Fulcher. An anti-A2 mAb413 (epitope: 484–509) (26) was kindly provided by Dr. Evgueni Saenko. The mAbs ESH4 and ESH8, with different C2 epitopes (residues 2303–2332 and 2248–2285, respectively), were purchased from American Diagnostica Inc. (Greenwich, CT, USA). FVIIa-specific inhibitor peptide E-76 (Bachem, Torrance, CA, USA) and recombinant lipidated TF (Innovin®; Dade Behring, Marburg, Germany) were purchased from the indicated vendors. The biotinylation of mAbC5 was prepared using *N*-hydroxysuccinimido-biotin reagent (Pierce). PL vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine (Sigma, St Louis, MO, USA) were prepared using *N*-octylglucoside (27). Protein concentrations were determined by the method of Bradford (28).

Anti-FVIII inhibitor antibodies

Thirteen anti-FVIII inhibitor alloAbs or autoAbs were obtained from Japanese patients with congenital severe haemophilia A or acquired haemophilia A, respectively. IgG fractions were prepared using protein A Sepharose (Amersham Biosciences, Uppsala, Sweden). $F(ab')_2$ fragments were prepared using immobilised pepsin-Sepharose (Pierce). The inhibitor titer of antibody $F(ab')_2$ was determined by the Bethesda assay by Kasper et al. (29). The kinetic patterns (type 1 and type 2 behaviours) of FVIII inactivation by these anti-FVIII antibodies were determined in one-stage clotting assays (12). Epitopes of these antibodies were performed by SDS-PAGE and Western blotting using isolated FVIII fragments (purified A1, A2, A3, C2, HCh and LCh, and thrombin-cleaved LCh fragments). Bound to FVIII fragments of anti-FVIII antibodies was detected by the addition of anti-human peroxidase-linked monoclonal antibody (The Binding Site Group Ltd, Birmingham, UK).

FVIII activation by FVIIa/TF

FVIII activity was measured in a one-stage clotting assay using FVIII-deficient plasma (Sysmex, Kobe, Japan) with an equipment of STart4 Hemostasis Analyzer® (Diagnostica Stago, Asnieres,

France). All reactions were performed at 37°C. FVIII activation by FVIIa/TF was performed as previously reported (21). FVIII (10 nM) was preincubated with the indicated concentrations of anti-FVIII antibodies or mAbs IgG $F(ab')_2$ for 1 hour (h), and then reacted with FVIIa/TF (1 nM) and PL (20 μM) in 20 mM HEPES, 150 mM NaCl, and 0.01% Tween 20 (HBS-buffer) containing 5 mM $CaCl_2$ and 0.1% bovine serum albumin. The final concentrations (f.c.) of inhibitors were adjusted to 2 BU/ml. Control $F(ab')_2$ was performed at the concentration of 500 nM. Samples were removed from the mixtures at the indicated times, and FVIIa/TF reaction was immediately terminated by the addition of the FVIIa-inhibitor E-76 (2.5 μM) and 200-fold dilution. The presence of FVIIa/TF, E-76, and anti-FVIII antibodies, in the diluted samples did not affect FVIII activity (<0.5%) in the coagulation assays (data not shown).

Cleavage of FVIII by FVIIa/TF

FVIII (10 nM) was preincubated with the indicated concentrations of anti-FVIII antibodies or mAbs IgG $F(ab')_2$ for 1 h, and then mixed with FVIIa/TF (1 nM) and PL (20 μM) in HBS-buffer containing 5 mM $CaCl_2$ (21). Aliquots were removed at the indicated times and the reactions were immediately terminated and prepared for SDS-PAGE by adding E-76 and SDS and by boiling for 3 minutes (min).

Electrophoresis and Western blotting

SDS-PAGE was performed using 8% gels at 150 V for 1 h. For Western blotting, protein samples were transferred to a polyvinylidene difluoride membrane using a Bio-Rad mini-transblot apparatus at 50 V for 2 h in buffer containing 10 mM CAPS, pH 11 and 10% (v/v) methanol. Protein bands were probed using unlabelled or labelled anti-A1 mAbC5, followed by a goat anti-mouse peroxidase-linked secondary mAb (MP Biomedicals, Aurora, OH, USA) or horseradish peroxidase-labelled streptavidin (Chemicon, Australia), respectively. Signals were detected using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA). Densitometric scans were quantitated using Image J 1.38 (National Institute of Health, Bethesda, MD, USA).

Results

Properties of anti-FVIII inhibitor alloAbs or autoAbs

Our goal in this study was to determine the effects of anti-FVIII inhibitor antibodies, in particular, the effects of antibodies with different recognising epitopes, on FVIIa/TF-catalysed activation and inactivation of FVIII. We chose anti-FVIII antibodies, therefore, based on the recognition of either the A2 or C2 domain of major inhibitor epitopes. Determination of the domain specificity of anti-FVIII antibodies was performed by Western blotting using isolated FVIII fragments (data not shown). Two cases bound to the HCh and A2, but any little reacted with either the A1 or LCh. Eleven cases bound to the (thrombin-cleaved) LCh and C2, but failed to react with the A1, A2, and A3. To confirm the specificity of these reactions, the antibodies of the former or the latter were preincubated with excess amounts of A2 or C2 in a fluid phase, prior to reaction with the HCh or LCh, respectively, followed by immunoblotting. The reactivities to the HCh of two cases were completely lost by the A2 domain, suggestive of the recognition of A2 epitope(s). Similarly, the reactivities to the LCh of 11 cases were lost competitively by the C2 domain, suggestive of the recognition of C2 epitope(s), although the contamination of other epitope could be completely excluded. The inhibitor titres of all cases, determined by the Bethesda assay, ranged from 6.3 to 440 BU/mg. The kinetic patterns of FVIII inactivation for 13 cases were also investigated (12). Two anti-A2 cases showed the type 1 pattern (named by cases 12 and 13). Seven of 11 anti-C2 cases showed the type 1 pattern (named by cases 1–7), and the other 4 cases showed the type 2 pattern (named by cases 8–11). Taken together, the properties of 13 anti-FVIII allo- or autoAbs and 3 anti-FVIII mAbs used in the present study are summarised in ▶Table 1.

Table 1: Characterisation of anti-FVIII inhibitor antibodies obtained from congenital or acquired haemophilia A patients and anti-FVIII mAbs.

Case	Type of anti-FVIII Ab	Epitope	Type of kinetics	Inhibitor titer (BU/mg)
1	alloAb	C2	1	151 ± 3
2	alloAb	C2	1	300 ± 7
3	autoAb	C2	1	13.6 ± 0.8
4	autoAb	C2	1	14.4 ± 1.0
5	autoAb	C2	1	440 ± 10
6	autoAb	C2	1	21.8 ± 1.8
7	autoAb	C2	1	42.3 ± 2.4
8	alloAb	C2	2	73.5 ± 3.2
9	autoAb	C2	2	18.3 ± 1.5
10	autoAb	C2	2	524 ± 18
11	autoAb	C2	2	6.3 ± 0.8
12	alloAb	A2	1	141 ± 6
13	alloAb	A2	1	29.8 ± 1.3
ESH4	mAb	C2	1	39 ± 2
ESH8	mAb	C2	2	10,000 ± 34
413	mAb	A2	1	59,500 ± 69