or type 2 inhibitors, respectively (7). Epitopes of autoAbs and haemophilic alloAbs have been found commonly in the A2, C2, or both domains of the FVIII molecule (8). Most autoAbs appear to be directed against a single domain rather than both domains, with anti-C2 antibodies being more common than anti-A2 antibodies (8). In contrast, most haemophilic alloAbs appear to recognise both domains. Anti-C2 type 1 antibodies inhibit FVIIIa binding to PL membranes (9, 10). The FVIII binding to PL and VWF is mutually exclusive (11), and antibodies have been shown to block binding to both PL and/or VWF (12, 13). Furthermore, anti-C2 type 2 antibodies interfere with FVIII activation mediated by thrombin and/or FXa (9, 10, 14).

Accurate measurements of blood coagulation *in vitro* are essential for the complete clinical assessment of clotting function. Conventional one-stage clotting tests (prothrombin time and activated partial thromboplastin time; APTT) are useful for routine laboratory examination, but they only partially reflect coagulation in a non-physiological environment and are based on the classical concepts of intrinsic and extrinsic cascade mechanisms. Discrepancies between coagulant activity and clinical phenotype in patients are often apparent, therefore. Recently, interest has focused on global coagulation assays, developed from a better understanding of the coagulation reaction involving tissue factor (TF)-triggered, cell-based mechanisms generating thrombin on activated platelets (15). Global tests of this nature such as the thrombin generation test (TGT) and clot waveform analysis have been established (16–18). We have further reported that our optimisation of these techniques provided a quantitative evaluation of clotting function in patients with very low levels of FVIII:C, and that various parameters closely correlated with clinical phenotype (18–20).

According to a retrospective survey, the severity of AHA is not directly associated with FVIII:C level (21), and AHA patients frequently present with life- or limb-threatening bleeding episodes that appear to be more pronounced than in congenital HA, although FVIII:C levels are similar in both. Hence, the clinical phenotype is often severe in AHA patients with moderate or even mildly deficient levels of FVIII:C. The reason(s) for this discrepancy in AHA remains to be clarified, however.

In the present study, patients with moderate-type HA (M-group), severe-type HA (S-group), AHA with type 1 (type 1) and with type 2 inhibitors (type 2) were investigated. We have demonstrated for the first time that coagulation function, assessed using global coagulation assays, was significantly more depressed in AHA with anti-C2 autoAbs compared to congenital HA, particularly in the S-group. We propose that one possibility for this difference is that the complex of FVIII and anti-C2 autoAbs indirectly interferes with FIXa-dependent FX activation due to steric hindrance. In addition, type 1 anti-C2 autoAbs prevented FVIII(a)-PL binding mechanisms, essential for the tenase complex, and type 2 anti-C2 autoAbs decreased FXa generation by inhibiting FVIII activation mediated by thrombin (and/or FXa). These distinct mechanisms might be associated with the exacerbated haemorrhagic symptoms in AHA.

Materials and methods

Reagents

An anti-FVIII A2 mAbJR8 was obtained from JR Scientific Inc. (Woodland, CA, USA). Anti-FVIII C2 mAbs, ESH4 and ESH8, recognising residues 2303–2332 and residues 2248–2285, respectively (12, 22), were purchased from American Diagnostica Inc. (Stamford, CT, USA). An anti-C2 alloAb was purified from plasma obtained in a severe HA patient with inhibitor. An anti-FIX mAb3A6 was prepared as previously reported (23). The biotinylation of mAb was prepared using *N*-hydroxysuccinimido-biotin (Pierce, Rockford, IL, USA). Recombinant lipidated TF (Innovin®; Dade Behring, Marburg, Germany), ellagic acid (Sysmex, Kobe, Japan), thrombin-specific fluorogenic substrate (Bachem, Bubendorf, Switzerland), and thrombin calibrator (Thrombinoscope, Maastricht, Netherlands) were obtained from the indicated vendors. Human thrombin, FV, FIXa, FX, FXa (Hematologic Technologies, Inc. Essex, VT, USA), recombinant hirudin (Calbiochem, San Diego, CA, USA), FXa substrate S-2222 and thrombin substrate S-2238 (Chromogenix, Milano, Italy), and plasma-derived FVIII-deficient plasma (George King Biomedical. Overland Park, KS, USA) were commercially purchased. PL vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, 30% phosphatidylethanolamine (Sigma) were prepared as previously described (24).

Proteins

Purified recombinant (r)FVIII preparations (Kogenate FS®) were a generous gift from Bayer Corp. Japan (Osaka, Japan). The A1, A2, HCh, LCh, and thrombin-cleaved LCh fragments were isolated from recombinant FVIII (25). The rA3 and rC2 proteins were purified as previously reported (26, 27). VWF was purified from FVIII/VWF concentrates (28). SDS-PAGE of the isolated subunits followed by staining with GelCode Blue Stain Reagent (Pierce) showed >95% purity (data not shown). Protein concentrations were measured using the Bradford method (29).

Patients' plasmas

Whole blood was obtained by venipuncture into tubes containing 1:9 volume of 3.8% (w/v) trisodium citrate. After centrifugation for 15 minutes (min) at 1,500 g, the plasmas were stored at –80°C, and thawed at 37°C immediately prior to the assays. Inhibitor titres were determined using the Bethesda assay (30). The kinetic pattern (type 1 or type 2 behaviour) of FVIII inactivation by anti-FVIII autoAbs was determined using one-stage clotting assays (7). Patients' plasmas were obtained from moderate-type HA (M-group, n=10, FVIII:C: 2.1 ± 0.9 IU/dl), severe-type HA (S-group, n=15, FVIII:C: <0.2 IU/dl), type 1 AHA (type 1, n=9, FVIII:C: <0.2 IU/

dl, 167 ± 175 BU/ml) and type 2 AHA (type 2, n=8, FVIII:C: 2.0 ± 1.9 IU/dl, 202 ± 120 BU/ml). The present studies were performed using blood samples obtained from patients diagnosed by our research group (Table 1) and enrolled in the Nara Medical University Hemophilia Program. All samples were obtained after informed consent following local ethical guidelines.

Anti-FVIII autoAbs

Anti-FVIII autoAb IgGs were purified from AHA plasma. IgG preparations were fractionated by affinity chromatography on protein G-Sepharose. $F(ab')_2$ fragments were prepared using immobilised pepsin-Sepharose (Pierce) (31). The FVIII domain(s) recognised by these antibodies were determined by SDS-PAGE and Western blotting using isolated FVIII fragments. The binding of anti-FVIII antibodies to FVIII fragments was detected using anti-human peroxidase-linked secondary antibody (Dako Japan, Tokyo, Japan). All autoAbs reacted predominantly or exclusively with the C2 domain in these immunoblot analyses using isolated FVIII fragments. Little reaction was evident with coagulant proteins other than FVIII (data not shown). All of these autoAbs, therefore, were regarded as anti-C2 autoAbs.

Clot waveform analysis

FVIII(a) activity was measured in one-stage clotting assay using FVIII-deficient plasma. APTT measurements were also performed using the MDA-II™ Hemostasis System (Trinity Biotech, Dublin, Ireland) with commercially available APTT reagent. The clot waveforms obtained were computer-processed using the commercial kinetic algorithm (18). The minimum value of the first derivative (min1), defining the maximum velocity of change in light transmission, was calculated as an indicator of the maximum velocity of coagulation achieved. The second derivative of the transmittance data (d^2T/dt^2) reflects the acceleration of the reaction at any given time point. The minimum value of the second derivative (min2) was also calculated as an index of the maximum acceleration of the reaction achieved. Since the minimum of min1 and min2 are derived from negative changes, we expressed the data as |min1| and |min2|, respectively. The clot time was defined as the time until the start of coagulation.

Thrombin generation test (TGT)

The calibrated automated TGT (Thrombinoscope) was performed as previously described (16, 20). Sample plasma (80 µl) was pre-incubated for 10 min with 20 µl of trigger reagent containing TF, PL, and ellagic acid (final concentration (f.c.) 0.5 pM, 4 µM, and 0.3 µM, respectively). Measurements were then commenced after the addition of 20 µl reagent containing $CaCl_2$ and fluorogenic substrate (f.c. 16.7 mM and 2.5 mM, respectively). The development of fluorescent signals was monitored using a Fluoroskan Ascent microplate reader (Thermo Fisher Scientific, Boston, MA, USA). Data analyses were performed using the manufacture's software, and the standard parameters; peak thrombin, time to peak, and endogenous thrombin potential (ETP), were derived.

Table 1: Properties of plasma samples and anti-C2 autoAbs obtained from AHA patients.

Case	FVIII:C (IU/dl)	FVIII:Ag (IU/dl)	Inhibitor (BU/ml)	Kinetic pattern	Recognition	
					Coagulant factor	Epitope*
1	<0.2	1.0	30.7	type 1	FVIII	C2
2	<0.2	<1.0	1,100	type 1	FVIII	C2
3	<0.2	<1.0	48.8	type 1	FVIII	C2,(A2)
4	<0.2	<1.0	110	type 1	FVIII	C2
5	<0.2	11.1	32.7	type 1	FVIII	C2,(A2)
6	<0.2	<1.0	135	type 1	FVIII	C2
7	<0.2	6.5	33.1	type 1	FVIII	C2
8	<0.2	<1.0	65.3	type 1	FVIII	C2
9	<0.2	11.2	36.8	type 1	FVIII	C2
10	1.1	1.0	8.2	type 2	FVIII	C2
11	1.2	1.0	33.0	type 2	FVIII	C2
12	1.2	29.0	7.9	type 2	FVIII	C2
13	1.4	1.0	860	type 2	FVIII	C2,(A2)
14	1.5	2.8	300	type 2	FVIII	C2
15	6.4	14.1	1.2	type 2	FVIII	C2
16	1.0	10.0	31.7	type 2	FVIII	C2
17	3.2	2.4	10.6	type 2	FVIII	C2

Type 1 or Type 2 antibodies inhibit FVIII:C either completely or incompletely at saturating concentrations. *: Cases 3, 5, and 13 reacted very faintly with the A2 domain.

FXa generation assay

FXa generation was performed at 37°C in 20 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM $CaCl_2$, and 0.01% Tween 20 (HBS) containing 0.1% bovine serum albumin (BSA).

Purified reagent-based assays

(i) FVIIIa/FIXa-dependent FXa generation (32) – FVIII (0.05 nM) was activated by thrombin (1 nM), and this reaction was terminated after 1 min by the addition of hirudin (0.5 unit/ml). After dilution, FXa generation was initiated by the addition of FIXa (1 nM), FX (150 nM), and PL (20 µM).

(ii) FVIII/FIXa-dependent FXa generation – FXa generation was initiated by the addition of FIXa (1 nM), FX (150 nM), PL (20 μM), and hirudin (0.5 unit/ml) to FVIII (0.05 nM) and continued for 30 min. In both assays, aliquots were removed at the indicated times to assess initial rates of product formation, and added to tubes containing EDTA. Initial rates of FXa generation were determined at 405 nm after the addition of S-2222.

Plasma-based assays

A commercial COATEST®SP FVIII kit (Chromogenix) was used according to the manufacture's instructions. Plasma samples were diluted five fold in HBS containing 0.1% BSA and were mixed with FIXa/FX/PL. FXa generation was initiated by the addition of $CaCl_2$, and the initial rates were determined at 405 nm after the addition of S-2765. Hirudin (1 unit/ml) was added to the samples to stop positive-feedback activation mediated by the generated thrombin.

FVIII competitive binding assay

FVIII (25 nM) in 10 mM Tris and 150 mM NaCl, pH7.4, was immobilised onto microtiter wells at 4°C overnight. After blocking with 5% BSA at 37°C for 2 hours (h), serial dilutions of anti-C2 autoAbs together with constant concentrations (10 μg/ml) of anti-C2 mAbESH4 or mAbESH8 were added to each well, and were further incubated for 2 h. Bound mAbESH4 or mAbESH8 was detected after 2-h incubation with horseradish peroxidase-conjugated anti-mouse IgG and the addition of *o*-phenylenediamine. The amount of nonspecific IgG binding without FVIII was <5% of the total signal. Specific binding was estimated by subtracting the amount of non-specific binding.

ELISA for FVIII binding to immobilised VWF or PL

Binding of FVIII to VWF or PL were examined as previously reported (28). VWF (40 nM) or PL (20 μM) was immobilised onto microtiter wells. After blocking with 5% BSA, FVIII (1 nM) was added onto the immobilised VWF well or PL well. Bound FVIII was detected using biotinylated anti-A2 mAbJR8 and horseradish peroxidase-labeled streptavidin. The amount of non-specific IgG binding without FVIII was <3% of the total signal. Specific binding was estimated by subtracting the amount of non-specific binding.

FVIII cleavage by thrombin or FXa

FVIII (10 nM) was preincubated with the indicated concentrations of anti-C2 autoAbs for 1 h. The mixtures were then incubated at 37°C with thrombin (5 nM) or FXa (0.3 nM) together with PL (20 μM) in HBS-buffer containing 5 mM $CaCl_2$. Aliquots were removed at the indicated times and the reactions were terminated and prepared for SDS-PAGE by adding SDS and boiling for 3 min. SDS-PAGE was performed using 8% gels at 150 V for 1 h, followed by Western blotting. Protein bands were probed using the indicated mAb followed by goat anti-mouse peroxidase-linked secondary mAb. Signals were detected using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA). Densitometric scans were quantitated using Image J 1.38.

Statistical analysis

The significant of the differences between each of AHA groups, congenital HA groups and samples with anti-C2 mAbs were determined by paired Student's t-test analysis.

Results

Global blood coagulation in moderate HA (M-group) and AHA-type 2

AHA-type 2 patients exhibited more severe haemorrhagic symptoms than the HA M-group, although similar levels of FVIII:C were recorded in one-stage clotting assays. The TGT has been recently developed to evaluate global coagulation function based on the principles of cell-based clotting, and we utilised this technique in this study. Although TF at low concentration is generally used as a trigger in the TGT, sensitive differences in coagulation function at low levels of FVIII:C (<~3 IU/dl) are not seen (20). We have reported, however, that the addition of small amounts of ellagic acid to the mixtures containing the low TF-trigger TGT had little effect on the lag-time (representing activation of the FVIIa/TF-induced extrinsic pathway) but provided higher peak thrombin and ETP measurements (representing the subsequent activation of the intrinsic pathway) (20). The modified TGT, therefore, reflected global coagulation sensitivity in the intrinsic pathway as well as the extrinsic, cell-based pathway, and enabled evaluation of coagulation function at very low levels of FVIII:C (low limit; <0.4 IU/dl).

This TGT was utilised in the present study and plasma samples were mixed with TF (0.5 pM), PL (4 μM), and ellagic acid (0.3 μM), followed by the addition of $CaCl_2$ and fluorogenic substrate (20). Representative thrombograms (*upper panels*) and the derived parameters (*lower panels*) in the M-group and type 2 AHA are illustrated in ▶ Figure 1A. The levels of peak thrombin and ETP obtained in type 2 were significantly decreased relative to those in the M-group, by ~2.6-fold (type 2/M-group: 61 ± 30/159 ± 50 nM, $p<0.01$; *panel* a) and by ~2.2-fold (1,310 ± 810/2,848 ± 620 nM, $p<0.01$; *panel* c), respectively. The time to peak was markedly prolonged by ~1.9-fold (32.2 ± 5.8/17.1 ± 2.0 min, $p<0.01$; *panel* b).

Global coagulations parameters in both groups were further

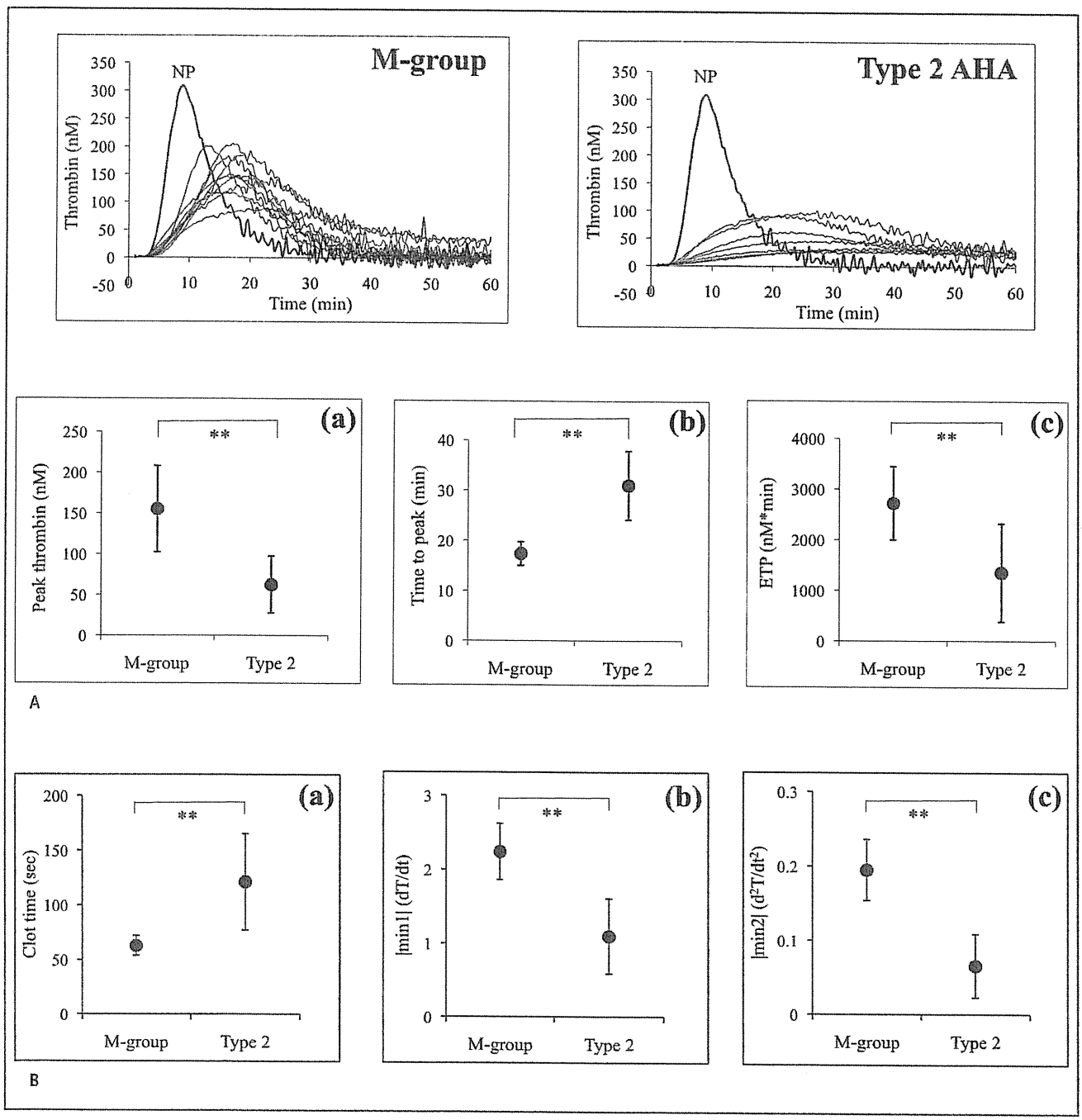


Figure 1: TGT and clot waveform analysis on patient's plasmas in the M-group and type 2 AHA. A) TGT-assay; upper panels: Plasma samples obtained from the M-group patients and type 2 AHA were preincubated with TF (0.5 pM), PL (4 μM) and ellagic acid (0.3 μM), followed by the addition of $CaCl_2$. Thrombin generation was measured as described in *Methods*, and representative TGT curves are illustrated. NP; control normal plasma. Lower panels: The peak thrombin (a), time to peak (b), and ETP (c) were derived from the TGT data obtained in upper panels. B) Clot waveform analysis; The APTT of patients' plasmas obtained from M-group and type 2 AHA were measured using the MDA-II™ system. The parameters clot time (a), |min1| (b), and |min2| (c) were derived from the clot waveform data as described in *Methods*. In all instances, results are shown as mean ± SD from at least five separate experiments. **p<0.01.

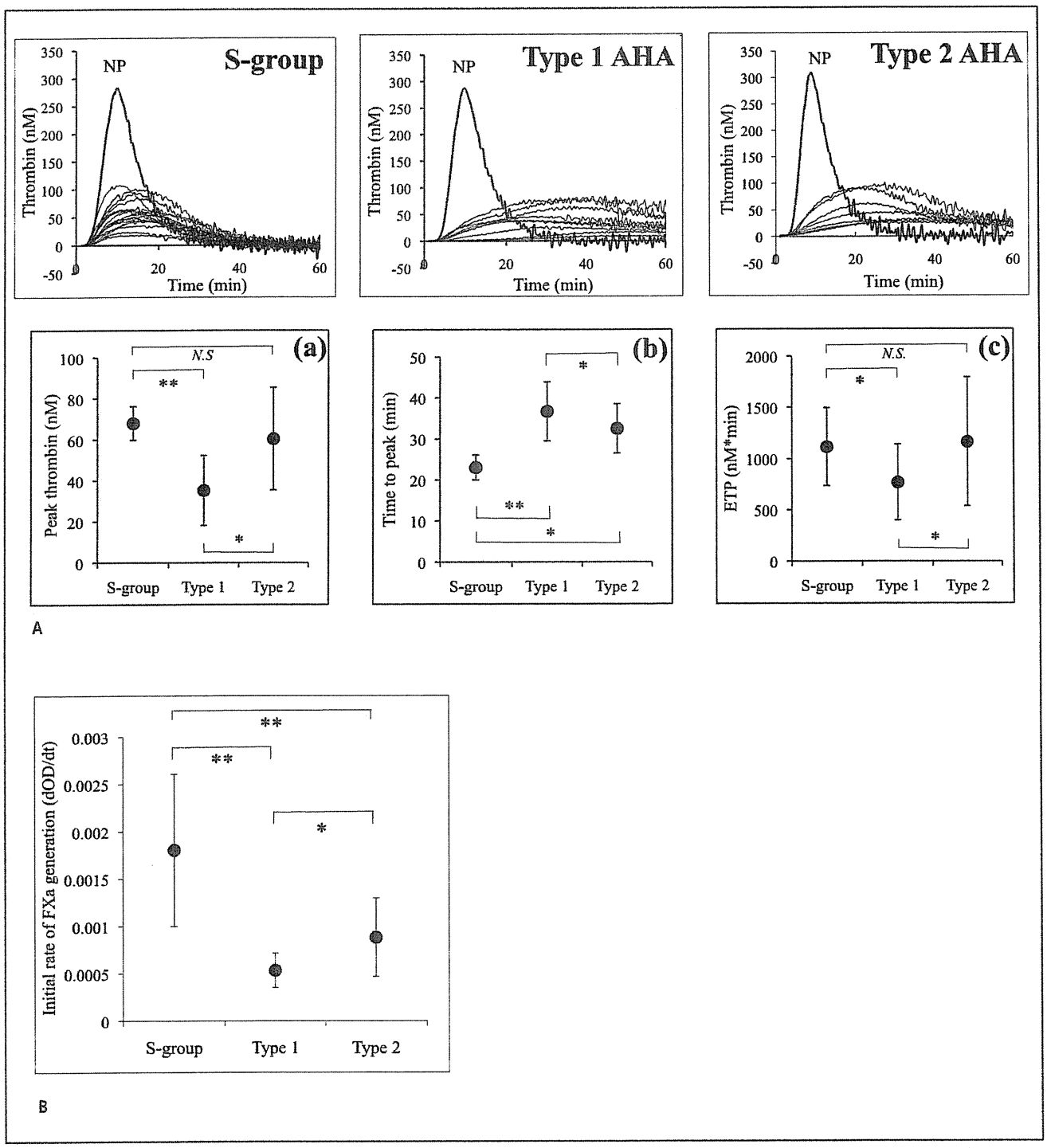


Figure 2: TGT and endogenous intrinsic FXa generation on patient's plasmas in the S-group, type 1, and type 2 AHA. A) TGT-assay; Upper panels: Patients' plasmas obtained from the S-group, type 1 AHA, and type 2 AHA were preincubated with TF (0.5 pM), PL (4 μM) and ellagic acid (0.3 μM), followed by the addition of $CaCl_2$. Thrombin generation was measured as described in *Methods*, and representative TGT curves are illustrated. NP; control normal plasma. Lower panels: The parameters of peak thrombin (a), time to peak (b), and ETP (c) were obtained from the TGT data shown in upper panels. B) Endogenous intrinsic FXa generation; Patients' plasmas obtained from S-group, type 1, and type 2 AHA were preincubated with FIXa/FX/PL mixture in the presence of hirudin, followed by the addition of $CaCl_2$ as described in *Methods*. FXa was measured using commercial reagents. The initial velocity rates of endogenous FXa generation are illustrated. In all instances, results are shown as mean ± SD from at least five separate experiments. The value of FVIII:C 1.0 IU/dl as a reference value was 5.04 ± 0.20 $x10^{-3}$. *p<0.05, **p<0.01, NS; no significance.

evaluated by clot waveform analysis using the MDA-II™ system (18). Unlike the TGT, this analysis reflects the process of fibrin formation. The data obtained from these waveforms are illustrated in ▶Figure 1B. The clot times in type 2 were prolonged by ~2.0-fold (121 ± 44/61 ± 8 seconds, p<0.05; *panel* a), compared to those in the M-group, and both |min1| and |min2| values were significantly decreased by ~2.1-fold, (1.1 ± 0.5/2.3 ± 0.3, p<0.01; *panel* b) and by ~3.1-fold (0.06 ± 0.03/0.19 ± 0.04, p<0.01; *panel* c), respectively. These results demonstrated that blood coagulation in type 2 was markedly more defective than in the M-group, despite similar FVIII:C levels (2.1 ± 0.9/2.0 ± 1.9 IU/dl, respectively). The findings were in keeping with the more severe haemorrhagic symptoms observed in type 2 relative to the M-group of patients.

Comparisons of coagulation function in severe HA (S-group) and AHA

More severe haemorrhagic symptoms are evident in the AHA patients compared to those in the S-group (FVIII:C<0.2%). These clinical differences were examined, therefore, using the TGT in these patients. Representative thrombograms from the S-group, type 1, and type 2 are illustrated in ▶Figure 2A (*upper panels*). The derived parameters are shown in the *lower panels*. The levels of peak thrombin and ETP in type 1 were markedly decreased by ~2-fold (type 1/S-group: 35.2 ± 14.1/68.0 ± 8.2 nM, p<0.01, *panel* a) and by ~1.5-fold (770 ± 310/1,115 ± 381 nM, p<0.05, *panel* c), respectively. The time to peak in type 1 was significantly prolonged by ~1.6-fold (36.7 ± 6.5/23.0 ± 3.0 min, p<0.01, *panel* b), compared to those in S-group. Similarly, in type 2, the time to peak was significantly delayed compared to that in S-group (32.5 ± 6.0/23.0 ± 3.0 min, p<0:05, *panel* b). These findings again provided strong evidence that the more serious clinical symptoms in AHA were related to the differences in global coagulation profiles, even though the FVIII:C in AHA were similar or slightly higher level than those in S-group. Surprisingly, thrombin generation in type 1 was moderately, but significantly more defective than in type 2 (p<0.05). It appeared, therefore, that coagulation function in the three groups was depressed in the order type 1, type 2, S-group.

Intrinsic FXa generation, corresponding to the upstream process of thrombin generation, was further examined to clarify the mechanism(s) of excessively defective thrombin generation in AHA. Plasma samples from each of the three groups were incubated with FIXa/FX/PL mixtures in the presence of hirudin (to eliminate thrombin reactions). $CaCl_2$ was added and endogenous intrinsic FXa generation was measured using the chromogenic assay. The initial rate of FXa generation was decreased in the order type 1, type 2, S-group (0.53 ± 0.18/0.88 ± 0.41/1.81 ± 0.78 x10^{-3}) with significant differences (▶Fig. 2B). These results were consistent with those obtained in the TGT, and further suggested that the discrepancies in coagulation function between AHA and S-group HA could be attributed to a significant decrease in the expression of intrinsic tenase complex activity (FVIIIa/FIXa/FX/PL).

Properties of anti-FVIII autoAbs in AHA

To further investigate the mechanism(s) by which the coagulation function in AHA was more defective than in the S-group, anti-FVIII autoAbs purified from AHA plasmas were characterised. FVIII levels and the basic properties of these autoAbs are summarised in ▶Table 1. Other coagulation factor activities in all cases were within the normal range (data not shown). SDS-PAGE and Western blotting using purified coagulation proteins revealed that all autoAbs reacted with FVIII alone. In particular, they all strongly reacted with the C2 domain, although some additionally reacted very faintly with the A2 domain.

The C2 domain is associated with interactions with VWF and PL (33). We examined, therefore, the effects of anti-C2 autoAbs on FVIII binding to VWF and PL in ELISA. In all type 1 cases examined the antibodies dose-dependently inhibited FVIII binding to VWF (by 64–87%) and PL (by 60–79%) at the maximum concentration of 50 μg/ml (▶Table 2), and the inhibitory effects were dose-dependent (data not shown). In contrast, in all type 2 cases the antibodies did not affect binding. Insufficient amounts of purified $F(ab')_2$ were obtained from some type 1 cases (cases 7–9) and type 2 cases (cases 16–17), however, and these individuals could not be investigated.

Different effects of anti-C2 autoAbs on thrombin-catalysed FVIII reactions

The conversion of FVIII to FVIIIa by thrombin is essential for the expression of intrinsic tenase activity (5), and one particular FVIII binding-region has been located within the C2 domain (34). We examined, therefore, the effects of anti-C2 autoAbs on thrombin-catalysed FVIII activation. FVIII (0.05 nM) was preincubated with varying amounts of AHA autoAbs. After incubation with thrombin for 1 min, the reaction was stopped by the addition of hirudin, and the reactant mixtures were diluted to completely exclude the inhibitory effects of autoAbs. FXa generation was initiated by the addition of FIXa (1 nM) and FX (150 nM) (▶Fig. 3A, *upper panel*). Results are summarised in ▶Table 2. All type 2 antibodies (50 μg/ml) decreased the peak levels of thrombin-mediated FVIII activation by 66–94%, and the inhibitory effects were dose-dependent. Type 1 autoAbs little affected these reactions (by <5%), however. In these experiments, the presence of anti-C2 autoAbs may have interfered with FXa generation and indirectly moderated thrombin-catalysed FVIII activation. To investigate this, therefore, we examined direct thrombin-catalysed FVIII cleavage in the presence of anti-C2 autoAbs. Proteolytic cleavage at Arg^{372} and Arg^{1689} is essential for generating FVIIIa activity (5). FVIII (10 nM) was preincubated with anti-C2 autoAbs (≤100 μg/ml), and was then activated by thrombin (5 nM), followed by SDS-PAGE and Western blotting using anti-A2 mAbJR8 (▶Fig. 3A, *lower panels*). All type 2 antibodies delayed the appearance of intact A2 during early-timed reactions (*panel* a). The inhibitory effects were dose-dependent by 61–73% (at 50 μg/ml), and were consistent with inhibition of

Case	Inhibition of FVIII binding to		Inhibition of thrombin-catalysed reaction of FVIII			Inhibition of FXa-catalysed reaction of FVIII			Competition of FVIII binding to	
	VWF	PL	Activation	Cleavage (%)		Activation	Cleavage (%)		ESH4	ESH8
	(%)		(%)	Arg372	Arg1689	(%)	Arg372	Arg1689	(%)	
Type 1										
1	67	72	<5	<5	<5	n.d.	n.d.	n.d.	78	12
2	86	64	<5	<5	<5	n.d.	n.d.	n.d.	69	15
3	64	60	<5	<5	<5	n.d.	n.d.	n.d.	73	<5
4	77	63	<5	<5	<5	n.d.	n.d.	n.d.	69	<5
5	72	69	<5	<5	<5	n.d.	n.d.	n.d.	63	<5
6	87	79	<5	<5	<5	n.d.	n.d.	n.d.	84	<5
Type 2										
10	<5	<5	84	70	80	68	72	>95	<5	69
11	<5	<5	81	69	72	92	66	>95	<5	77
12	<5	<5	66*	64	35	63*	72	>95	<5	64
13	<5	<5	83	61	40	95	70	>95	14	86
14	<5	<5	73	63	68	83	79	>95	19	84
15	<5	<5	94	73	80	59	54	>95	<5	67

Reactions of anti-C2 autoAbs were examined as described in *Methods*. Data represent the inhibitory effects (%) at concentrations of 50 µg/ml for all cases except for case 12* (70 µg/ml). Insufficient amounts of $F(ab')_2$ were available from cases 7–9 (Type 1) and cases 16–17 (Type 2). n.d.: not determined.

Table 2: Properties of anti-C2 autoAbs obtained from AHA patients.

cleavage at Arg^{372} (*panel* b). Similarly, inhibition of cleavage at Arg^{1689} (by 35–80%) was observed with all type 2 antibodies (▶Table 2). These cleavage patterns appeared to be little affected (by <5%) by type 1 antibodies, consistent with the results of FVIII activation.

FXa-catalysed FVIII activation was also investigated, as a target for inhibitory effect of anti-C2 autoAbs. It was difficult, however, to assess FVIIIa-dependent FXa generation in the presence of purified FXa as an activator of FVIII. Consequently, FVIII-dependent FIXa-catalysed FXa generation was evaluated. This assay depended on the positive-feedback mechanism(s) by which FIXa-catalysed FXa generation mediated FVIII activation. FVIII (0.05 nM) was preincubated with varying amounts of autoAbs, followed by the addition of FIXa (1 nM), FX (150 nM), PL (20 µM), and hirudin to initiate FXa generation (▶Fig. 3B). All type 2 antibodies (50 µg/ml) diminished the level of FXa generation by 59–95%, and the inhibitory effects were dose-dependent (*upper panel*). To directly examine FXa-catalysed FVIII proteolysis, FVIII (10 nM) was mixed with autoAbs (50 µg/ml) prior to incubation with FXa (0.5 nM) and PL (20 µM) (▶Fig. 3B, *lower panels*). All type 2 antibodies inhibited cleavage at Arg^{372} by 54–79% in a timed-dependent manner, and the inhibitory effects were dose-dependent (*panels* a and b). Cleavage at Arg^{1689} was also completely inhibited (by >95%) by all type 2 antibodies (▶Table 2). The inhibitory effects of type 1 antibodies could not be determined precisely, however, since these antibodies directly inhibited FVIII(a)-PL interaction.

Coagulation function in AHA-model reconstituted with FVIII/anti-C2 mAb

The inhibitory properties of anti-C2 type 1 and type 2 autoAbs obtained in the present study were similar to those reported by Meeks et al. (9, 10). To investigate whether the pivotal C2 epitopes of our autoAbs overlapped with those of anti-C2 mAbESH4 or mAbESH8, representing typical type 1 or type 2 behaviour, respectively, competitive inhibition for FVIII binding were examined. All type 1 autoAbs significantly competed with ESH4 binding to FVIII by 63–84%, but competed with ESH8 binding by <5–15%. In contrast, all type 2 autoAbs competed with ESH8 binding to FVIII by 64–86%, but competed with ESH4 binding by <5–19%. These findings indicated that anti-C2 type 1 and type 2 autoAbs contained the C2 epitopes identified in ESH4 and ESH8, respectively (▶Table 2).

We compared, therefore, the coagulation parameters in *in vitro* models of AHA, constructed with exogenous anti-C2 mAbs (ESH4 and ESH8), with those of the S-group. FVIII (10 IU/dl) was preincubated with ESH4 (80 µg/ml) or ESH8 (20 µg/ml), and residual FVIII:C was adjusted to <0.2 and ~2 IU/dl, respectively, similar to the levels in AHA patients. The mixtures were added to FVIII-deficient plasma and utilised in the TGT-assay (▶Fig. 4A and B). The time to peak in the AHA-models with ESH4 and ESH8 (48.8 ± 2.0/47.6 ± 2.4 min, $p<0.01/p<0.05$, respectively) were prolonged compared to the S-group (43.0 ± 1.6 min), reflecting decreased coagulation function in the presence of ESH4/ESH8. These findings

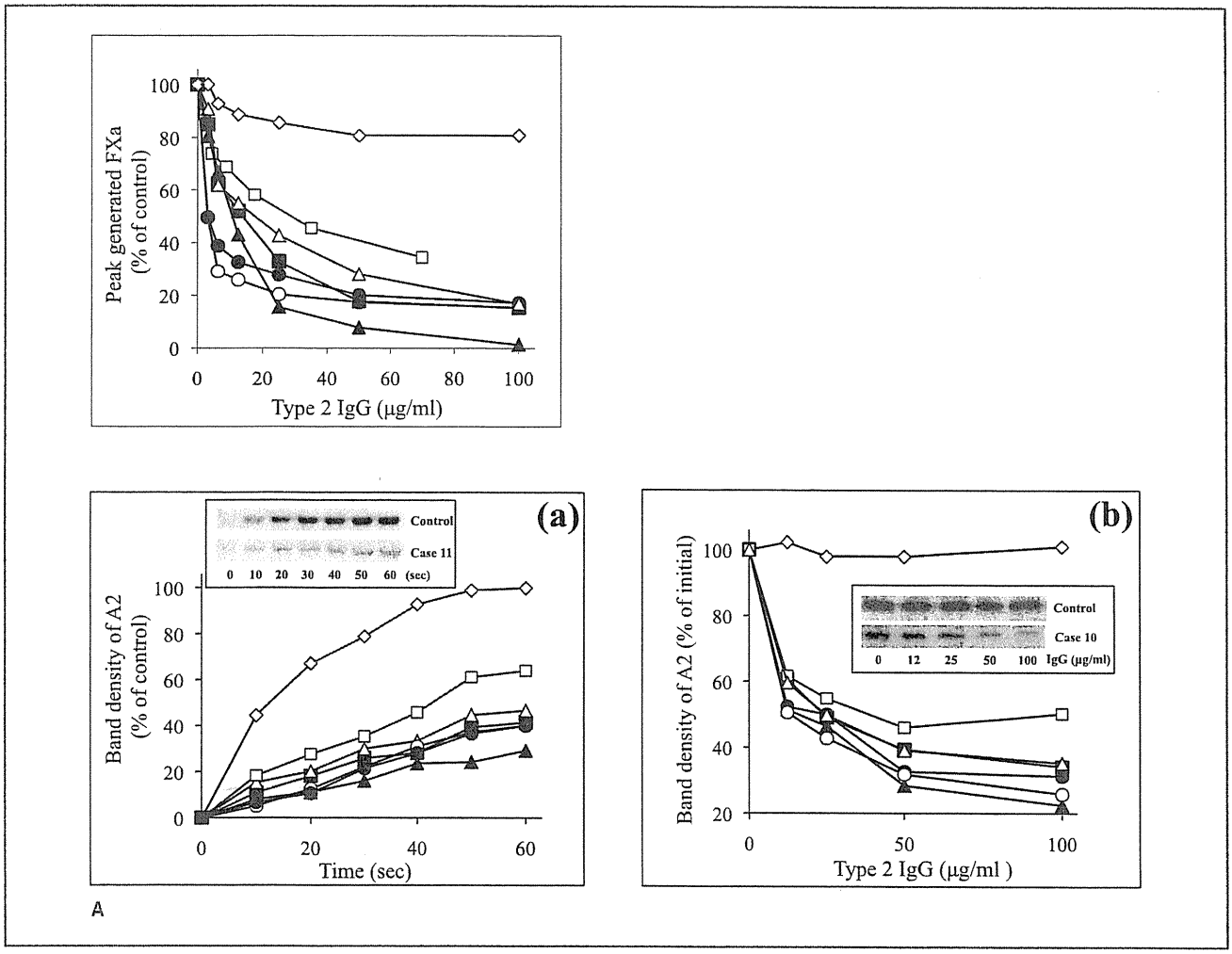
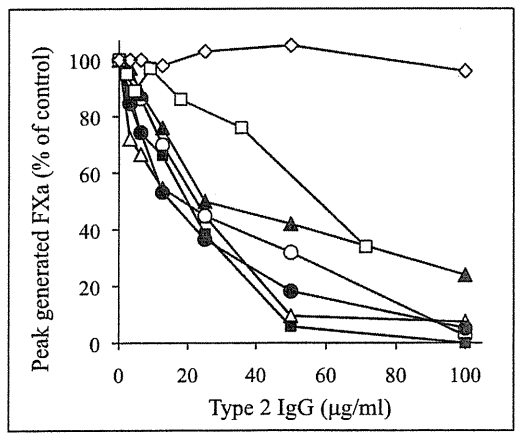
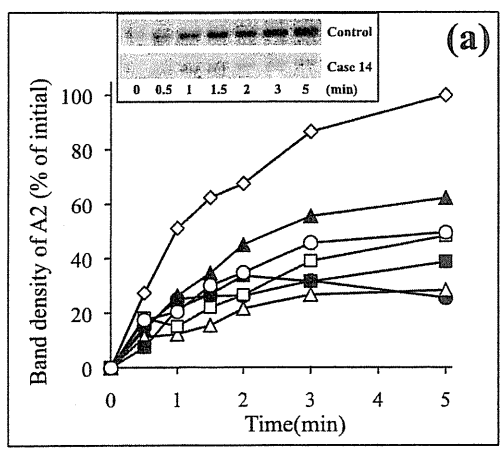
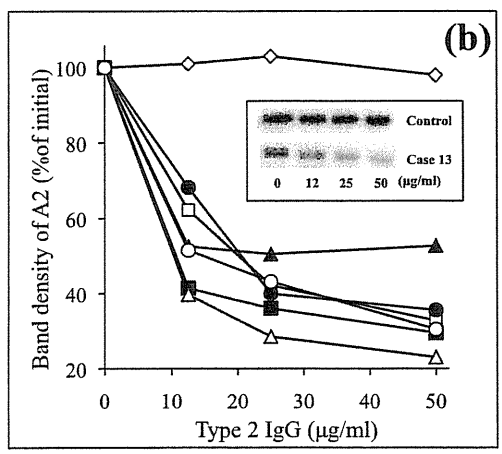


Figure 3: Effects of type 2 anti-C2 autoAbs on thrombin- or FXa-catalysed activation of FVIII. A) Thrombin reaction; Upper panel: FVIII (0.05 nM) was activated by thrombin (1 nM) for 1 min. After the addition of hirudin and dilution, FXa generation was initiated by the addition of FIXa (1 nM), FX (150 nM), and PL (20 μM). Various concentrations of type 2 autoAbs were preincubated with FVIII prior to adding thrombin, followed by adding hirudin to terminate the thrombin reaction. The rate of FXa generation without anti-C2 autoAb was regarded as 100%. In all instances, results are shown as mean from at least five separate experiments. Lower panels: (a) FVIII (10 nM) was mixed with type 2 autoAbs (100 μg/ml) for 1 h, followed by incubation with thrombin (5 nM) for the indicated times. Samples were run on 8% gel followed by Western blotting using anti-A2 mAbJR8. Band density of A2 at 1 min after adding thrombin with normal $F(ab')_2$ was regarded as 100%. (b) FVIII (10 nM) was mixed with various concentrations of type 2 autoAbs for 1 h, followed by incubation with thrombin (5 nM) for 1 min. Samples were run on 8% gel followed by Western blotting using anti-A2 mAb. Band density of A2 after adding thrombin in the absence of type 2 autoAbs was regarded as 100%. B) FXa reaction; Upper panel: FVIII (0.05 nM) was incubated with various concentrations of type 2 autoAbs for 1 h. FXa generation was initiated by the addition of FIXa (1 nM), FX (150 nM), PL (20 μM) in the presence of hirudin for 30 min. The rate of endogenous intrinsic FXa generation in the absence of autoAb was regarded as 100%. In all instances, results are shown as mean from at least five separate experiments. Lower panels: (a) FVIII (10 nM) was mixed with type 2 autoAbs (50 μg/ml) for 1 h, followed by incubation with FXa (0.5 nM) and PL (20 μM) for the indicated times. Samples were run on 8% gel followed by Western blotting using anti-A2 mAb. Band density of A2 at 5 min after FXa incubation with normal $F(ab')_2$ was regarded as 100%. (b) FVIII (10 nM) was mixed with various concentrations of type 2 autoAbs for 1 h, followed by incubation with FXa (0.5 nM) and PL (20 μM) for 5 min. Samples were run on 8% gel followed by Western blotting using anti-A2 mAb. Band density of A2 by FXa in the absence autoAbs was regarded as 100%. The symbols used are: ○; case 10, ●; case 11, □; case 12, ■; case 13, Δ; case 14, ▲; case 15, ◊; normal $F(ab')_2$.







B

Figure 3: Continued

were in keeping with those observed using anti-C2 AHA plasmas in thrombin and FXa generation assays (see ▶Fig. 2A and B). No significant differences were observed between ESH4 and ESH8 in these assays, however.

Effect of anti-FIX mAb on TGT in FVIII-deficient plasma

Our findings suggested that the additional decrease of coagulation function in AHA relative to S-group could be attributed to the markedly decreased activity of the intrinsic tenase complex. Since intrinsic tenase activity in S-group HA depends on FIXa-catalysed FX activation, we hypothesised that inhibition of FIXa-induced FX activation could have mediated the significantly greater decrease in tenase activity observed in AHA. We examined, therefore, the effects of anti-FIX mAb on thrombin generation in FVIII-deficient plasmas (▶Fig. 5). Control experiments demonstrated that the addition of anti-FVIII alloAb (10 BU/ml) to FVIII-deficient plasmas resulted in similar TGT parameters compared to its absence, confirming complete FVIII deficiency in the plasma samples. Furthermore, the addition of anti-C2 autoAbs to FVIII-deficient plasma little affected thrombin generation (data not shown), confirming that the effects of anti-C2 autoAbs in AHA patients depended on the presence of FVIII.

In addition, anti-FIX mAb3A6 (10 BU/ml) was incubated with FVIII-deficient plasmas, and TGT assays performed as above. Peak thrombin levels in the presence of anti-FIX mAb were significantly more decreased (~1.3-fold) than its absence (8.0 ± 0.5/10.4 ± 0.6 nM, $p<0.05$, ▶Fig. A). Similarly, ETP was more depressed (~1.5-fold) in the presence of anti-FIX mAb than its absence (100 ± 10/144 ± 15 nM, $p<0.05$, ▶Fig. 5C), and the time to peak was prolonged by ~1.3-fold (32.7 ± 1.9/25.0 ± 1.8 min, $p<0.05$, ▶Fig. 5B). These findings were similar to those obtained with native AHA plasmas, and the results were consistent with the concept that the exacerbated haemorrhagic symptoms in AHA with anti-C2 autoAbs, compared to S-group, could be related, in part, to indirect

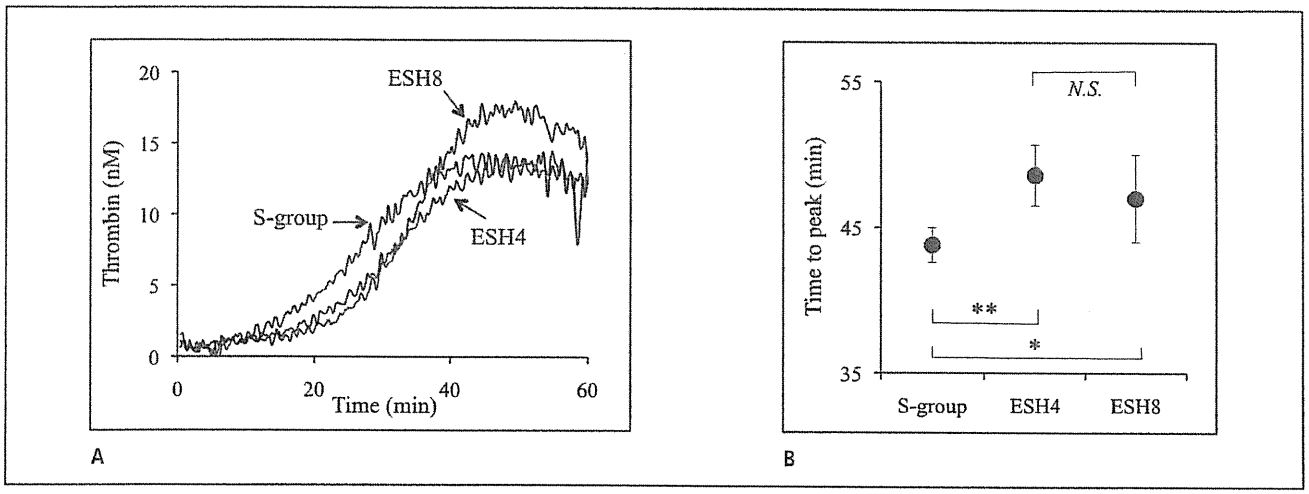


Figure 4: Coagulation function in *in vitro* AHA-models reconstituted with FVIII and anti-C2 mAb. A) FVIII (0.1 nM) was mixed with anti-C2 mAb ESH4 (80 μg/ml) or mAbESH8 (20 μg/ml) for 1 h prior to incubation with FVIII-deficient plasma. Samples were mixed with TF (0.5 pM), PL (60 μM), and ellagic acid (0.3 μM), followed by the addition of $CaCl_2$. Thrombin generation was measured as described in *Methods*. Representative TGT curves were illustrated. B) The time to peak obtained from the TGT is shown in (A). Data are shown as mean ± SD for data from at least five separate experiments. *p<0.05, **p<0.01, NS; no significance.

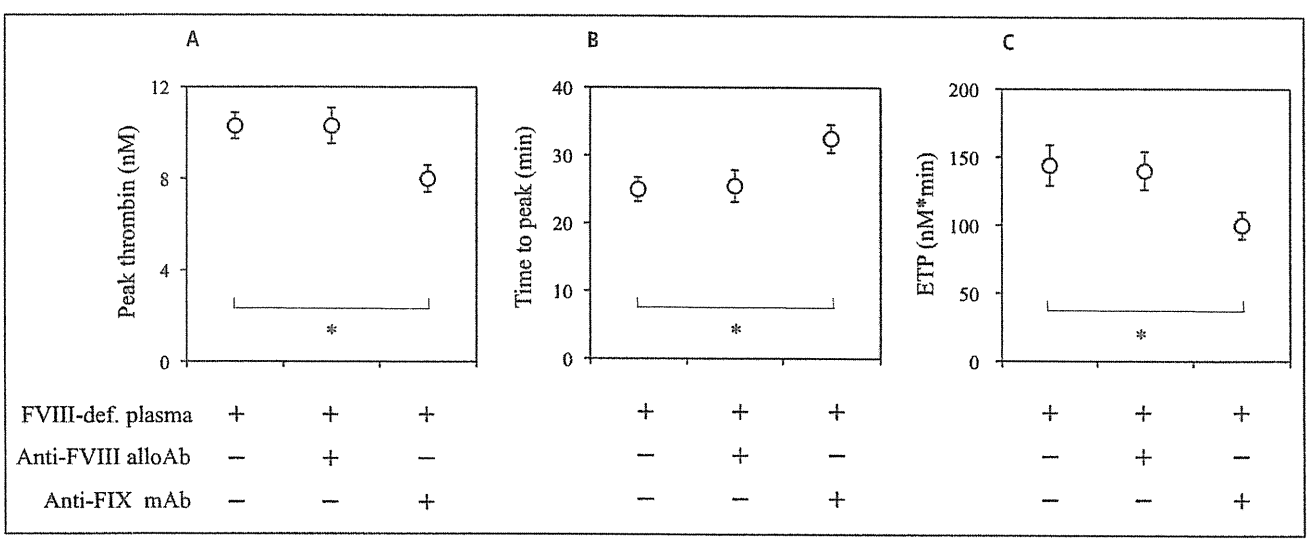


Figure 5: Effect of anti-FIX mAb on the thrombin generation in FVIII-deficient plasmas. FVIII-deficient plasma was preincubated with or without anti-C2 alloAb (10 BU/ml) for 1 h, and was reacted with or without anti-FIX Ab (10 BU/ml) for 1 h. These samples were reacted with TF (0.5 pM), PL (60 μM), and ellagic acid (1.8 μM), followed by the addition of $CaCl_2$. Thrombin generation was measured as described in *Methods*. A-C) Parameters of peak thrombin, time to peak, and ETP obtained from TGT. In all instances, results are shown as mean ± SD from at least five separate experiments. *p<0.05.

inhibition of FIXa-catalysed FX activation due to steric hindrance in the presence of the FVIII-anti-C2 autoAbs complex.

Discussion

The reason(s) why haemorrhagic symptoms in AHA are more severe than those in severe HA, although FVIII:C levels are similar, have not been clarified. The present findings suggest for the first time, that the mechanisms involved in these circumstances could possibly be attributed to the inhibition of FIXa-mediated FX activation by disturbances (steric hindrance) on the tenase complex in the presence of FVIII-anti-C2 autoAb complexes.

AHA antibodies with anti-A2 epitopes were not available for study, and all anti-FVIII autoAbs used in this study recognised the C2 domain. All anti-C2 Type 1 antibodies blocked FVIII binding to VWF and PL, but did not affect FVIII activation by thrombin. In

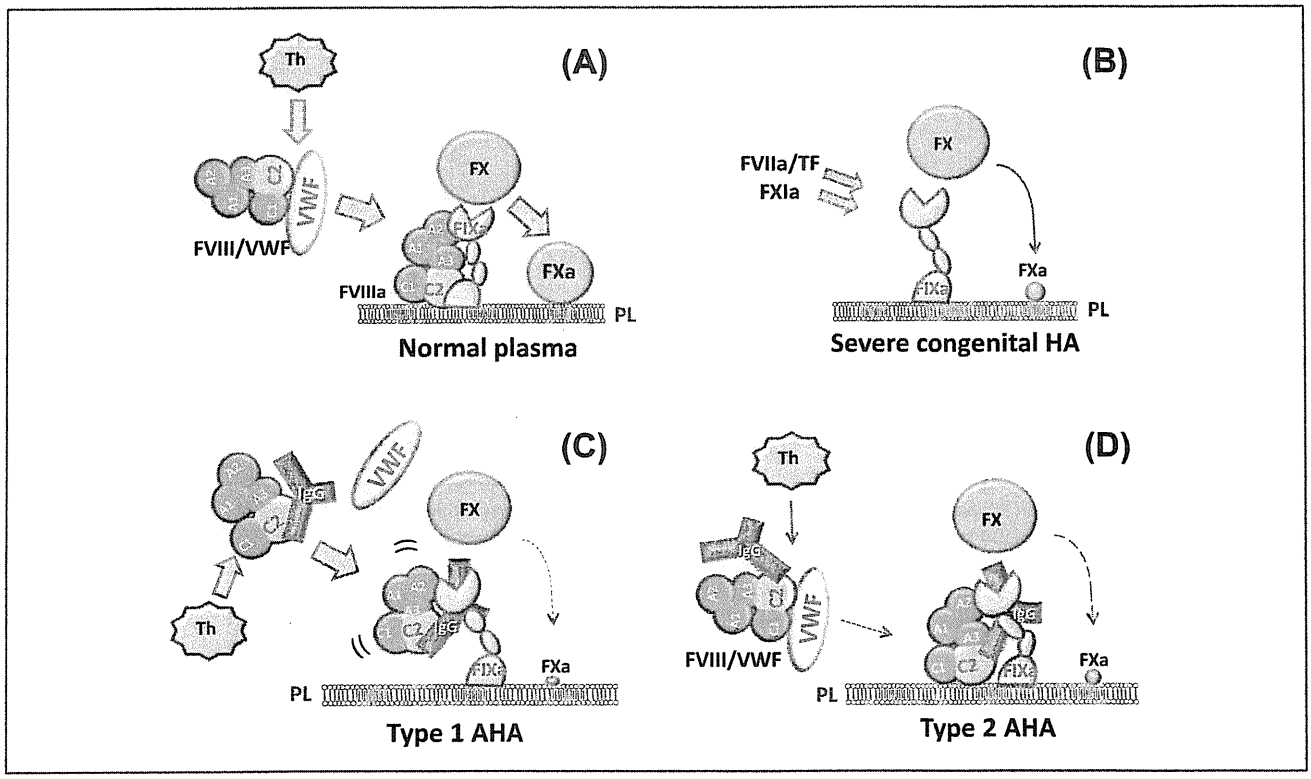


Figure 6: A putative coagulation mechanism for the intrinsic tenase complex in patients from the S-group, anti-C2 type 1, and type 2 AHA. In normal plasmas, free FVIIIa is generated from FVIII/VWF by thrombin, followed by FVIIIa/FIXa-dependent FX activation on PL micelles (A). In severe HA, FIXa alone generates FXa from FX very slowly (panel B). In both type 1 and type 2 AHA, anti-C2 IgG-FVIIIa complexes interfere with FIXa-catalysed FX activation on PL by steric hindrance. In type 1 cases, this complex fails to bind to PL, and the tenase assembly is unstable (C). In type 2 cases, although anti-C2 IgG significantly blocks thrombin-induced FVIII activation, small amounts of the FVIIIa-IgG complex bind to PL, and consequently trace amounts of tenase assembly is formed (D).

contrast, all anti-C2 type 2 antibodies inhibited FVIII activation by thrombin, but did not affect FVIII binding to VWF and PL. These anti-C2 properties were similar to those reported by Meeks et al. (9, 10), and were representative of the classical and non-classical anti-C2 antibodies respectively. In addition, PL concentrations did not affect the difference between both groups in thrombin and FXa generation and binding assays (data not shown). SDS-PAGE and Western blotting analysis revealed that the inhibition of thrombin-catalysed FVIII activation by anti-C2 Type 2 was attributed to delayed cleavage at Arg^{372} and Arg^{1689}. It was of additional interest, that mAbESH8 with type 2 epitopes did not affect FVIII cleavage by thrombin at Arg^{372} (and Arg^{1689}) (data not shown, [35]), and the findings might have reflected a novel inhibitory mechanism for anti-C2 autoAb inhibitors. The C2 domain is structurally juxtaposed to the A1 domain (36), and inhibition of cleavage by anti-C2 type 2 may have been due to a polyclonal, steric effect of the anti-C2 autoAbs, although inhibition caused by the coincident presence of an anti-A2 autoAb (37) could not be excluded. We have recently demonstrated an interaction between the C2 domain (residues 2228–2240) and the FIXa Gla domain in the tenase complex (38). In the current studies, however, neither type 1 nor type 2 anti-C2 autoAbs inhibited C2 binding to FIXa (data not shown), suggesting that these antibodies had little direct affect on FVIIIa-FIXa interactions in the tenase complex.

Thrombin generation in AHA was significantly less than that in severe HA. Furthermore, intrinsic FXa generation in AHA, reflecting processes upstream of thrombin generation, was decreased relative to that in severe HA. The anti-C2 antibodies little inhibited prothrombinase activity (data not shown), it appeared, therefore, that critical differences between AHA and severe HA in the intrinsic tenase complex contributed to the clinical findings, and that these differences centered on the effects of anti-C2 autoAbs on FVIIIa, FIXa, FX, and PL interactions. In normal tenase reactions (▶ Fig. 6A), FVIII in complex with VWF, is converted to FVIIIa by thrombin and dissociated from VWF (39). FIXa (activated by FVIIa/TF and/or FXIa) together with FVIIIa, activates FX on PL-membrane surfaces, resulting in FXa generation. In severe HA in the absence of FVIIIa cofactor (▶ Fig. 6B), FX is slowly converted to FXa by FIXa. Our studies demonstrated that FIXa-mediated FX activation in the presence of anti-FIX mAb, or in AHA-models constructed with anti-C2 mAbs, was less effective than that in severe HA. Based on these data, therefore, we propose a putative mechanism for the markedly decreased coagulation function in AHA with anti-C2 autoAbs. We suggest that the anti-C2 autoAbs,

complexed with FVIIIa, indirectly interfere with the association between FIXa and FX on PL-membrane surfaces by steric hindrance. Consequently, FIXa-mediated activation of FX in these patients is depressed to a greater extent than in severe HA (▶Fig. 6C and D).

The assays of thrombin and FXa generation showed that critical coagulation functions in AHA type 1 were lower than those in type 2, and experiments using AHA-models containing anti-C2 mAbs with type 1 and 2 behaviour (ESH4 and ESH8) demonstrated a similar tendency. Both native anti-C2 type 1 autoAbs and ESH4 inhibit FVIII binding to VWF and PL, and this inhibition of VWF-binding would lead to significantly decreased levels of FVIII:C (2). Furthermore, although FVIII-IgG complexes can be completely activated by thrombin, the tenase complex failed to bind to PL-membranes in these circumstances, and the conformation of this complex would likely be extremely unstable (▶Fig. 6C). In contrast, our experiments with native anti-C2 type 2 autoAbs and ESH8 demonstrated that FVIII binding to VWF or PL was little inhibited. It appeared, therefore, that these autoAbs significantly inhibited FVIII activation by thrombin, but that the relatively small amounts of FVIIIa-IgG complex formed bound to PL, facilitating trace amounts of tenase assembly (▶Fig. 6D). Nevertheless, as with type 1 antibodies, indirect disturbances (steric hindrance) mediated by FVIIIa-IgG complexes would have inhibited FIXa-induced FX activation. We speculate, therefore, that differences in the inhibitory mechanisms between type 1 and type 2 antibodies might have contributed to the observations that coagulation parameters were depressed in the order type 1, type 2, and S-group patients. Further studies are required to clarify these mechanisms.

In view of our findings that the excessive decrease in coagulation function in AHA could be due to indirect inhibition of FIXa-dependent FX activation, it might be expected that the clinical severity in patients with severe FIX-deficiency (haemophilia B, HB) might be more pronounced than in those with severe HA. In this context, it is also noteworthy that thrombin generation *in vitro* in FIXa-deficient plasmas with undetectable FIX:C (the lowest limit of detection in our laboratory is <0.2 IU/dl (18)) was significantly lower than in severe HA (unpublished observation). It is well known, however, that the clinical symptoms in severe HA are more marked than in severe HB (40, 41). The reasons for these findings remain unclear, but it may be that additional mechanism(s) underlie the AHA phenotype. For example, FX may be sequestered in a non-functional complex with FVIII-anti-C2 autoAbs in AHA. Further investigations are required to clarify these mechanisms.

The current investigations have introduced a putative mechanism for the excessive clinical haemorrhagic state in AHA, although further studies are required to support this conclusion, and to clarify the clinical differences between different types of AHA. Nevertheless, treatment of AHA in patients with high titre inhibitors has historically involved the use of coagulation-bypassing agents. Meeks et al. (9) suggested, however, that administration of high-doses of FVIII should be considered more actively for patients with AHA anti-C2 type 2 inhibitors, but not in those with type 1 inhibitors. Their conclusion was based on the findings that the activity of high-titer type 2 inhibitors could be neutralised by increasing dosages of FVIII, and it may be that in the presence of low concentrations of exogenous FVIII, anti-C2 IgGs, complexed with FVIII, indirectly disturb the association between FIXa and FX. At high doses of exogenous FVIII, inhibitory activity could be completely neutralised, and unbound (free) FVIII would be available to participate in tenase assembly. Type 1 patients failed to respond to high-dose FVIII, however, and our present data are not totally consistent with those findings. The challenging observations warrant further investigation.

What is known about this topic?

- Acquired haemophilia A (AHA) is caused by the development of factor (F)VIII autoantibodies (autoAbs).
- AHA results in more serious haemorrhagic symptoms than in congenital severe HA, but the reason(s) remain unknown, however.

What does this paper add?

- Coagulation functions, assessed using the global coagulation assays, were significantly more depressed in AHA with anti-C2 autoAbs relative to congenital HA.
- As one of putative mechanism(s), we proposed that the FVIII/anti-C2 autoAb complexes appeared to interfere with FIXa-dependent FX activation indirectly due to steric hindrance.
- In addition, the anti-C2 autoAbs with type 1 behavior prevented FVIII(a)-phospholipid binding mechanisms, essential for the tenase complex, and those with type 2 behaviour decreased the FXa generation by inhibiting thrombin-catalysed FVIII activation, suggesting that these distinct mechanisms could be associated with the exacerbated haemorrhagic symptoms in AHA.

Acknowledgement

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Conflict of interest

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

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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

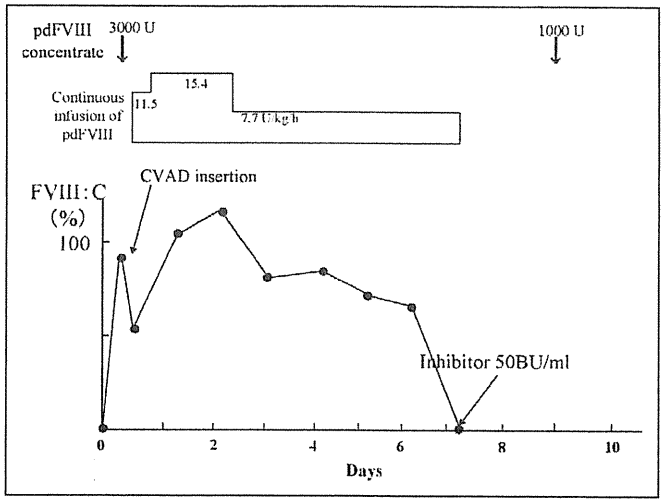

Figure 1. Clinical course of case 1. The FVIII:C after the infusion of 3000 U pdFVIII was 88% (expected 140%). Subsequently, continuous infusion of FVIII concentrate was administered. The patient required the dose of 7.7 to 15.4 U/kg per h to maintain the target levels of circulating FVIII:C. Seven days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

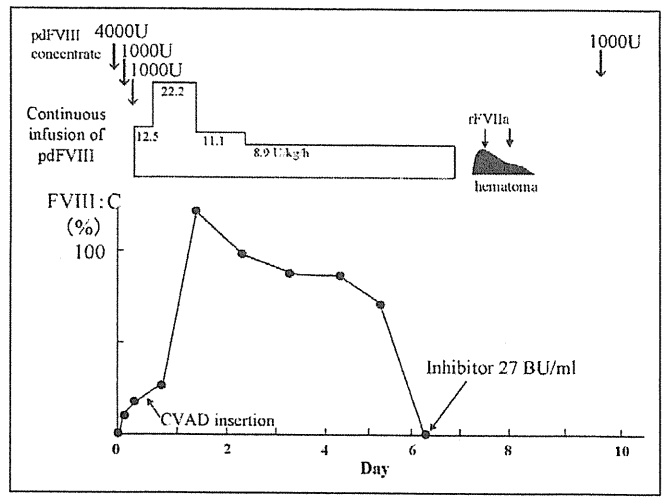

Figure 2. Clinical course of case 2. The FVIII:C in case 2 after the infusion of 3000 U pdFVIII was only 9.4% (expected 128%). An additional 1000 U pdFVIII raised the FVIII:C to 16%. A further 1000 U pdFVIII was given and surgery performed immediately. The operation was completed without untoward bleeding. 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.

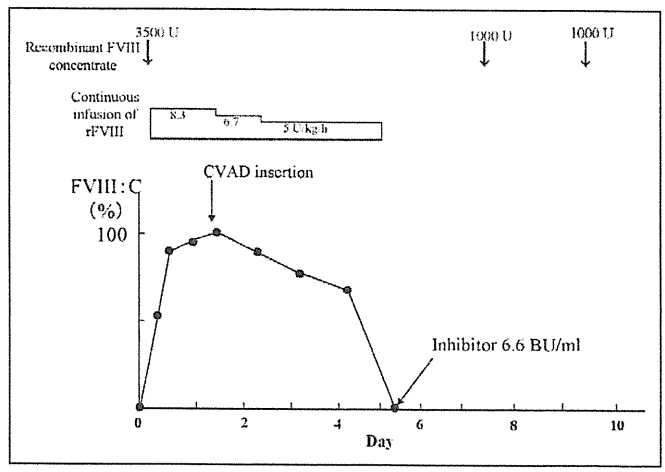

Figure 3. Clinical course of case 3.
The plasma level of FVIII:C in case 3 after the infusion of 3500 U rFVIII was 52% (expected 120%). The bolus infusion was followed by continuous infusion of at a dose of 8.3 U/kg per h and 12 hours later the plasma level of FVIII:C was 90%. Central venous access device 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.

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.

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

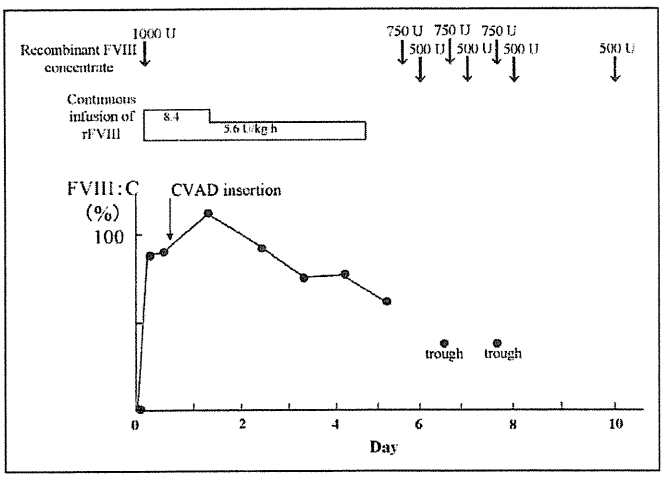

Figure 4. *Clinical course of case 4.*
The plasma level of FVIII:C in case 4 after the infusion of 1000 U rFVIII was 80% (expected 109%). The bolus infusion was followed by continuous infusion at a dose of 8.4 U/kg per h. Twelve hours later, the plasma level of FVIII:C was 90%, and the surgery commenced. 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

This submission is an original work and not previously published in any substantial part, not under consideration of publication elsewhere, and submitted to CATH for consideration of publication.

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The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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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).