Table 1 Number of exposures to trial products

	Vatreptacog alfa	rFVIIa	Total*
Number of patients	46	17	51
Exposure days† for trial product, *N* (%)			
1	25 (54.3)	15 (88.2)	24 (47.1)
2	13 (28.3)	2 (11.8)	15 (29.4)
3	7 (15.2)	–	7 (13.7)
4	–	–	4 (7.8)
5	1 (2.2)	–	1 (2.0)
Total dose (μg kg^{-1})			
Mean	86	214.8	
Minimum; maximum	5; 459	85; 528	
Total number of doses			
Mean	3.57	2.41	4.02
Minimum; maximum	1; 15	1; 6	1; 15

rFVIIa, recombinant FVIIa. *Total exposure days is not a sum of the numbers for vatreptacog alfa and rFVIIa directly, because, for example, if a patient had one exposure to vatreptacog alfa and one to rFVIIa, he had two exposures in total, and is included under the two row for total exposure days. †Exposure days = total number of days in which patients were treated with trial product.

Evaluation of safety: adverse events, including immunogenicity

Overall, vatreptacog alfa was well tolerated, with a low frequency of adverse events in all dose groups (Table 2). Most adverse events were related to concomitant illnesses and the underlying disease (hemophilia) and consequences thereof. The type and frequency of adverse events reported following exposure to vatreptacog alfa were comparable to the well-established adverse event profile of rFVIIa.

The majority of adverse events were rated as mild, and were judged by the investigator as unlikely to be related to the trial products. Three episodes of errors during reconstitution and administration of trial products (5 μg kg^{-1} vatreptacog alfa), and one episode of discomfort (after 90 μg kg^{-1} rFVIIa) were evaluated as being related to the trial products.

Fifteen serious adverse events were reported. All events started more than 2 weeks (range, 16–677 days) after treatment with trial product, and were evaluated as not being related to the trial product as judged by the investigator, DMC, and sponsor.

One thrombotic event was reported among patients exposed to trial product during the trial. In a 23-year-old patient exposed to vatreptacog alfa, a deep vein thrombosis of the right superficial femoral vein was reported as a serious adverse event with an onset 199 days after administration of 10 μg kg^{-1} vatreptacog alfa. The event, which occurred shortly after the patient suffered from cholecystitis requiring intensive care and after he had received alternative hemostatic medication according to local standard care, was judged as being unrelated to vatreptacog alfa exposure by the investigator, DMC, and sponsor.

A total of five patients were withdrawn from the trial because of adverse events, including three misdosing events (tier 1), one episode of elevated alanine aminotransferase (ALAT) level (tier 3), and the above-described venous thrombosis event (tier 2). No clinical symptoms were reported in relation to the dosing errors or the elevated ALAT value, which was present predose and was assessed as resulting from chronic hepatitis C. No formation of antibodies against vatreptacog alfa or rFVIIa was observed in any patients exposed to trial product.

Evaluation of safety: laboratory assessments

No safety concerns were revealed by any of the laboratory parameters investigated in the trial (troponin, hematology, including platelet count, biochemistry, including ALAT, and coagulation-related parameters).

No clinically relevant differences between predose and postdose values or differences between treatment groups were observed for platelet count (predose range, 126–474 × 10^9; 12-h postdose range, 127–502 × 10^9) or fibrinogen (predose range, 0.8–5.7 g L^{-1}; postdose range, 1.0–7.4 g L^{-1}; reference range,

Table 2 Overview of adverse events

	Vatreptacog alfa, *N* (%), *E*						rFVIIa, *N* (%), *E*
	5 μg kg^{-1}	10 μg kg^{-1}	20 μg kg^{-1}	40 μg kg^{-1}	80 μg kg^{-1}	Total	
Total bleeds	16	19	16	16	10	77	19
Adverse events	8 (50.0), 10	5 (26.3), 8	5 (25.0), 5	3 (18.8), 5	0 (0), 0	20 (26), 28	10 (52.6), 11
Serious adverse events*	2 (12.5), 3	3 (15.8), 5	2 (12.5), 2	2 (12.5), 2	0 (0), 0	9 (11.7), 12	2 (10.5), 3
Adverse events with onset within 7 days postdose	6 (37.5), 7	3 (15.8), 3	3 (18.8), 3	1 (6.3), 3	0 (0), 0	13 (16.9), 16	8 (42.1), 8
Possibly/probably related adverse events	3 (18.8), 3	0 (0.0), 0	0 (0), 0	0 (0), 0	0 (0), 0	3 (3.9), 3	1 (5.3), 1
Adverse events leading to withdrawal	3 (18.8), 3	1 (5.3), 1	0 (0), 0	0 (0), 0	0 (0), 0	4 (5.2), 4	1 (5.3), 1

E, number of adverse events; *N*, number of bleeds with an adverse event; %, proportion of bleeds with adverse event; rFVIIa, recombinant FVIIa. Non-serious adverse events include events occurring from the initial dose of trial product (for treatment of bleed) until 7 days after the initial dose. Serious adverse events include all events collected from the first administration of trial product to the end of patients' participation in the trial. *All serious adverse events had an onset more than 2 weeks (range, 16 days to almost 2 years) after treatment with trial product, and were evaluated as not related to the trial product as judged by the investigator, DMC, and sponsor.

2–4 g L^{-1}). No evidence of consumption of platelets or fibrinogen was apparent in any patients.

Postdose changes of D-dimers (Fig. 2A), F_{1+2} (Fig. 2B), PT and aPTT were considered to be consistent with the expected pharmacologic effect of both vatreptacog alfa and rFVIIa on the coagulation system.

Pharmacokinetic profile of vatreptacog alfa

Thirty patients had assessments of pharmacokinetic data, including a total of 42 bleeds treated in dose tier 3, 4, or 5. The FVIIa activity in plasma during the first 3 h after the initial intravenous dose of trial product is shown in Fig. 3. After the end of vatreptacog alfa infusion, FVIIa activity declined in an exponential way. A three-fold to four-fold higher peak activity was seen for 80 μg kg^{-1} vatreptacog alfa as compared with 90 μg kg^{-1} rFVIIa. At 1 h after the initial dose, mean plasma activities of vatreptacog alfa (20, 40 and 80 μg kg^{-1}) were below the levels obtained after a single dose of 90 μg kg^{-1} rFVIIa.

The total exposure during the initial 30 min ($AUC_{0-30\ min}$) and 3 h (AUC_{0-t}) postdose increased with increasing dose level of vatreptacog alfa, indicating dose linearity. The estimates of AUC_{0-t} and corresponding 95% confidence intervals did not indicate any statistically significant deviations from dose linearity for vatreptacog alfa.

The clearance of vatreptacog alfa was approximately three times faster than the clearance of rFVIIa.

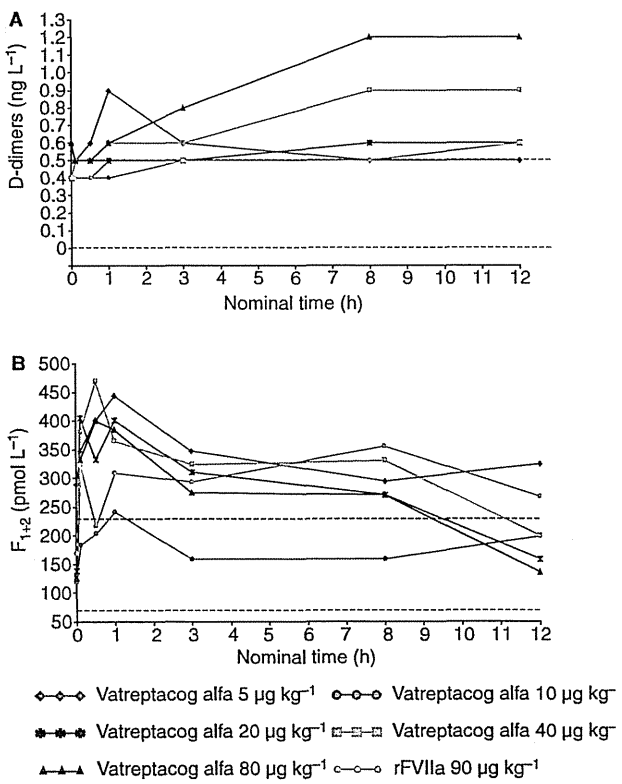

Fig. 2. Coagulation-related parameters of thrombin formation and fibrinolysis (A) Mean levels of D-dimers (ng L^{-1}) by dose. (B) Mean levels of F_{1+2} (pmol L^{-1}) by dose. Horizontal lines indicate the normal reference range. rFVIIa, recombinant FVIIa.

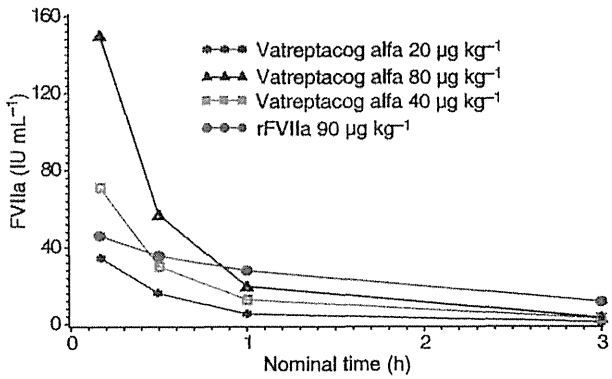

Fig. 3. Mean single-dose pharmacokinetic profiles of FVIIa activity vs. time for vatreptacog alfa (20, 40 or 80 μg kg^{-1}) and recombinant FVIIa (rFVIIa) (90 μg kg^{-1}).

Evaluation of efficacy

Ninety-five bleeds were included in the evaluation of efficacy (Fig. 1).

For the majority of joint bleeds (86/95 bleeds), effective and sustained control was obtained with the per-protocol one to three doses of trial product(s) (Table 3).

Overall, 98% of the joint bleeds were controlled successfully with vatreptacog alfa in a combined evaluation of 20–80 μg kg^{-1} dose groups, as compared with 90% of bleeds treated with rFVIIa (90 μg kg^{-1}).

The number of doses needed to obtain hemostatic control decreased with increasing dose of vatreptacog alfa, 40% of bleeds being effectively treated with a single dose of 80 μg kg^{-1} vatreptacog alfa (Table 3).

Discussion

Vatreptacog alfa was well tolerated in actively bleeding hemophilia patients with inhibitors enrolled in the current trial, with a low frequency of adverse events and no safety concerns being observed at any dose level. In particular, no immunogenic or thrombotic events related to the trial product were reported. In addition, no dose relationship was evident with respect to the incidence or nature of the adverse events reported following exposure to vatreptacog alfa. Notably, the type and frequency of adverse events reported were similar to the well-established safety profile of rFVIIa in patients with hemophilia. Hence, no adverse events specific to vatreptacog alfa were identified in the trial setting.

In vatreptacog alfa, three amino acids have been substituted vs. rFVIIa. Therefore, the formation of antibody against vatreptacog alfa was closely monitored. In the current trial, 45 patients received vatreptacog alfa and were followed with

Table 3 Treatment efficacy as evaluated by number of doses of trial product administered to control bleeds within 9 h from initial dose

	Vatreptacog alfa, *N* (%)					rFVIIa, *N* (%)
	5 μg kg^{-1}	10 μg kg^{-1}	20 μg kg^{-1}	40 μg kg^{-1}	80 μg kg^{-1}	90 μg kg^{-1}
Total bleeds	15	19	16	16	10	19
Treatment successes*	12 (80.0)	16 (84.2)	16 (100)	15 (93.8)	10 (100)	17 (89.5)
Bleeds controlled with a single dose	3 (20.0)	3 (15.8)	2 (12.5)	5 (31.3)	4 (40.0)	6 (31.6)
Bleeds controlled with two doses	5 (33.3)	9 (47.4)	7 (43.8)	6 (37.5)	4 (40.0)	4 (21.1)
Bleeds controlled with three doses	4 (26.7)	4 (21.1)	7 (43.8)	4 (25.0)	2 (20.0)	7 (36.8)

rFVIIa, recombinant FVIIa. *Bleeds successfully controlled with one to three doses of trial product.

antibody measurements for up to 28 days after the last trial product administration. The mean total number of vatreptacog alfa doses received during the trial was 3.6, ranging from 1 to 15. No antibody development was detected in any patients exposed to vatreptacog alfa in this trial. It should be borne in mind, however, that patients could receive vatreptacog alfa for a maximum of five bleeds with up to three doses per bleed, which may not be sufficient for the evaluation of immunogenic risk. To further evaluate the potential immunogenic risk of vatreptacog alfa, repeated assessment of potential antibody formation will be performed in future clinical trials.

One patient experienced a thrombosis of the right superficial femoral vein 199 days after administration of 10 μg kg^{-1} vatreptacog alfa, which was judged by the investigator, DMC and sponsor as being unrelated to the trial product. It should also be noted that his event occurred shortly after cholecystitis requiring intensive care, and after the patients had received hemostatic medication according to local standard care for treatment of a large iliopsoas bleed. Vatreptacog alfa exerts its pharmacologic action on activated platelets at the site of injury [10], and is not capable of directly activating platelets [8], so its action is limited to the site of injury. In addition, vatreptacog alfa is susceptible to the same plasma inhibitors (TF pathway inhibitor [TFPI] and antithrombin [AT]) as FVIIa, and, when bound to TF, is inhibited similarly to rFVIIa. In the absence of TF, vatreptacog alfa is inhibited more readily by TFPI and AT than rFVIIa. Approximately 70% of vatreptacog alfa is eliminated in the initial phase, with a half-life of ~ 20 min. This rapid elimination may contribute to a low risk of thromboembolic events in patients with hemophilia following exposure to or treatment with vatreptacog alfa. As the trial included first exposure of vatreptacog alfa in bleeding patients with hemophilia, a cautious dose-escalation trial was chosen, and the trial population comprised relatively young patients (age range, 12–69 years; mean, 28 years) without any known risk factors for thrombosis/arterial thrombosis. Therefore, assessment of the thrombogenic risk of vatreptacog alfa will be further evaluated in future trials.

The pharmacokinetic data obtained in bleeding patients with hemophilia and inhibitors are consistent with the pharmacokinetic profile of vatreptacog alfa previously reported in healthy subjects [14]. The FVIIa activity declined with a biexponential decay pattern, with initial rapid elimination being followed by a less rapid elimination phase. Dose proportionality could be concluded within the dose range studied (20–80 μg kg^{-1}). The pharmacokinetic profile obtained after intravenous administration of vatreptacog alfa was distinctly different from that obtained after the same dose of rFVIIa. As expected, the initial postdose concentration was significantly increased after administration of vatreptacog alfa. An approximately three-fold to four-fold higher initial activity for the 80 μg kg^{-1} dose than for 90 μg kg^{-1} rFVIIa was shown at 10 min postdose. At 1 h after injection of trial product, the mean plasma activities of vatreptacog alfa were below the levels obtained after rFVIIa administration. The clearance of vatreptacog alfa was approximately three times faster than the clearance of rFVIIa, as reflected in the initial half-life of vatreptacog alfa. The shorter half-life and faster clearance of vatreptacog alfa may be explained by the greater inhibition of vatreptacog alfa by AT, as suggested by recent studies [19,20].

Non-clinical data have shown that vatreptacog alfa displays increased procoagulant activity on activated platelets, resulting in a normalization of thrombin generation and formation of a stable clot [10,11]. Furthermore, animal data obtained in a mouse hemophilia model [12,13] showed that vatreptacog alfa efficiently shortened the bleeding time and reduced blood loss. This current trial expands upon these findings, showing a high efficacy rate of vatreptacog alfa in controlling acute joint bleeds at all dose levels studied, with an apparent dose relationship being observed for vatreptacog alfa efficacy endpoints.

The observed 90% efficacy rate in the rFVIIa comparator group is consistent with data from published clinical trials and experience, supporting the appropriateness of the trial design and the trial population.

In summary, a favorable safety profile of vatreptacog alfa with a low frequency of adverse events was observed in the patient population studied in this trial. In addition, preliminary efficacy evaluation showed a high efficacy rate of vatreptacog alfa, with 98% of joint bleeds being successfully controlled in a combined evaluation of the 20–80 μg kg^{-1} dose groups. However, as the trial was not powered for efficacy, assessment of the relative efficacy of vatreptacog alfa will need to be verified in a larger confirmatory trial.

On the basis of the clinical data obtained in the current trial and the pharmacologic characteristics, vatreptacog alfa may represent an improved option for the treatment of acute bleeding episodes in hemophilia patients with inhibitors.

Addendum

S. Ehrenforth: made substantial contributions to the conception and design of the trial; E. V. De Paula, K. Kavakli, J. Mahlangu, Y. Ayob, S. R. Lentz, M. Morfini, L. Nemes, S. Z. Šalek, M. Shima, J. Windyga, S. Ehrenforth, and A. Chuansumrit: performed research/contributed to the acquisition of data; E. V. De Paula, K. Kavakli, J. Mahlangu, Y. Ayob, S. R. Lentz, M. Morfini, L. Nemes, S. Z. Šalek, M. Shima, J. Windyga, S. Ehrenforth, and A. Chuansumrit: collected data; E. V. De Paula, K. Kavakli, J. Mahlangu, Y. Ayob, S. R. Lentz, M. Morfini, L. Nemes, S. Z. Šalek, M. Shima, J. Windyga, S. Ehrenforth, and A. Chuansumrit: analyzed and interpreted the data; E. V. De Paula and S. Ehrenforth: co-wrote the manuscript; E. V. De Paula, K. Kavakli, J. Mahlangu, Y. Ayob, S. R. Lentz, M. Morfini, L. Nemes, S. Z. Šalek, M. Shima, J. Windyga, S. Ehrenforth, and A. Chuansumrit: critically reviewed the manuscript for important intellectual content; E. V. De Paula, K. Kavakli, J. Mahlangu, Y. Ayob, S. R. Lentz, M. Morfini, L. Nemes, S. Z. Šalek, M. Shima, J. Windyga, S. Ehrenforth, and A. Chuansumrit: approved the final version of the manuscript. E. V. De Paula, the lead author, assumes full responsibility for the integrity and interpretation of the data.

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Disclosure of Conflict of Interests

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Participating principal investigators and sites.

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nature medicine

A bispecific antibody to factors IXa and X restores factor VIII hemostatic activity in a hemophilia A model

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Hemophilia A is a bleeding disorder resulting from coagulation factor VIII (FVIII) deficiency. Exogenously provided FVIII effectively reduces bleeding complications in patients with severe hemophilia A. In approximately 30% of such patients, however, the 'foreignness' of the FVIII molecule causes them to develop inhibitory antibodies against FVIII (inhibitors), precluding FVIII treatment in this set of patients[1–3]. Moreover, the poor pharmacokinetics of FVIII, attributed to low subcutaneous bioavailability and a short half-life of 0.5 d, necessitates frequent intravenous injections[3–5]. To overcome these drawbacks, we generated a humanized bispecific antibody to factor IXa (FIXa) and factor X (FX), termed hBS23, that places these two factors into spatially appropriate positions and mimics the cofactor function of FVIII. hBS23 exerted coagulation activity in FVIII-deficient plasma, even in the presence of inhibitors, and showed *in vivo* hemostatic activity in a nonhuman primate model of acquired hemophilia A. Notably, hBS23 had high subcutaneous bioavailability and a 2-week half-life and would not be expected to elicit the development of FVIII-specific inhibitory antibodies, as its molecular structure, and hence antigenicity, differs from that of FVIII. A long-acting, subcutaneously injectable agent that is unaffected by the presence of inhibitors could markedly reduce the burden of care for the treatment of hemophilia A.

About 1 in 10,000 males suffer from hemophilia A[6]. Approximately half of all patients with hemophilia A are classified as having severe disease[7], defined as <1% of normal FVIII activity, and such individuals typically experience at least one bleeding episode every month without preventive intervention[3,8]. Because patients with moderate hemophilia A (1–5% of normal activity) experience far fewer bleeding episodes, patients with severe disease are routinely given prophylactic supplementation of FVIII to keep the factor's activity to 1% or above, which effectively reduces joint bleeds, leading to better joint status and quality of life[3,8].

Despite these advantages, routine supplementation with FVIII has two major drawbacks aside from its expense: the development of inhibitors and the need for frequent venous access for FVIII injection. Inhibitors precluding the use of FVIII make it difficult to control hemorrhaging because alternative treatment agents (such as recombinant activated factor VII and activated prothrombin complex concentrates) have shorter half-lives, cost more than FVIII and are not always effective[9–11]. Eradication of inhibitors with high doses of FVIII is currently being attempted, but the process is very expensive and does not always work[12]. The need for frequent venous access is also problematic, particularly when treating pediatric patients at home[13], and it negatively affects both the implementation of and adherence to the supplementation routine. Therefore, a new agent that resolves these two drawbacks inherent to the current therapeutic use of FVIII has the potential to markedly improve the treatment possibilities for individuals with severe hemophilia A.

FVIII functions as a cofactor only when activated by either thrombin or factor Xa (FXa). The resultant factor VIIIa (FVIIIa) consists of the A1 subunit, the A2 subunit and the light chain (**Fig. 1a**)[14]. The light chain and the A2 subunit of FVIIIa respectively bind the light chain (K_d = 15 nM) and the heavy chain (K_d ≈ 300 nM) of FIXa[15,16]. The A1 subunit of FVIIIa binds the heavy chain of FX (K_d = 1–3 μM)[17]. These binding properties contribute to FVIII's cofactor activity, enhancing the catalytic rate constant of FIXa and the interaction between FIXa and FX[14].

Recombinant monoclonal antibodies, with not only antagonistic activity but also agonistic[18], catalytic[19] or allosteric activity[20], have been extensively studied for their therapeutic applications[21]. Furthermore, bispecific antibodies, which recognize two different antigens, have been applied not only to simply neutralize two different antigens but also to recruit effector cells against the target cells[22] and to co-ligate two different antigens on the same cell[23]. We hypothesized that a bispecific IgG antibody recognizing FIXa with one arm and FX with the other could place FIXa and FX in spatially appropriate positions, as FVIIIa does, and promote FIXa-catalyzed FX activation (**Fig. 1b**).

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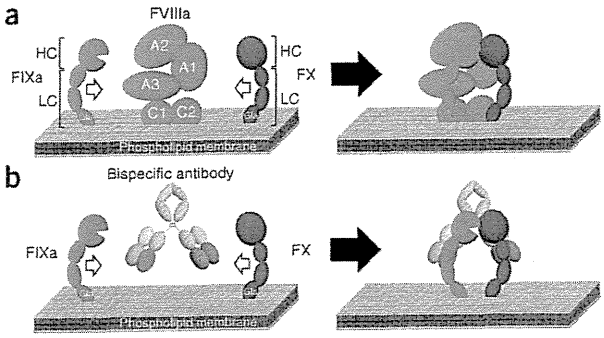

Figure 1 Schematic illustrations of the action of FVIIIa or of a bispecific antibody as a cofactor promoting the interaction between FIXa and FX. (**a**) FVIIIa consists of the A1 subunit, the A2 subunit and the light chain (A3, C1 and C2 subunits). FVIIIa forms a complex with FIXa and supports the interaction between FIXa and FX through its binding to both factors on the phospholipid membrane. HC, heavy chain; LC, light chain. (**b**) Bispecific antibody binding to FIXa and FX would promote the interaction between FIXa and FX on the phospholipid membrane and exert FVIII-mimetic activity. FVIIIa binds the phospholipid membrane via its C1 and C2 subunits; FIXa and FX bind the phospholipid membrane via their Gla domains. The illustrations describe the concept of the study only and do not necessarily indicate precise molecular structures and positions.

In support of this possibility, the distance between the FIXa- and FX-binding sites of FVIIIa[24] is similar to that between the two antigen-binding sites of human IgG[25]. In addition, antibody binding to the appropriate epitope of FIXa would mimic the allosteric properties of FVIIIa and enhance the catalytic activity of FIXa[14,16].

A bispecific antibody mimicking FVIII function could be an ideal agent for overcoming the two major drawbacks of current treatment. First, a bispecific antibody with a completely different molecular structure, or antigenicity, from FVIII would neither elicit inhibitors in patients with hemophilia A nor be neutralized by inhibitors that were already present. Moreover, humanized antibodies recently approved for clinical use have shown a low incidence of immunogenicity (<5%)[26]. Second, IgG antibodies have long plasma half-lives of 2 or 3 weeks and high subcutaneous bioavailability in humans, enabling once-a-month or less frequent treatment without venous access[27].

To create this type of bispecific antibody, we first generated various FIXa- and FX-specific monoclonal antibodies by immunizing animals with human FIXa and FX, respectively. We next used the genes encoding these monoclonal antibodies to generate bispecific IgG antibodies in which one arm would recognize FIXa and the other would recognize FX, as described in the Online Methods, and screened approximately 40,000 of these antibodies by an enzymatic assay for FX activation in the presence of FIXa and phospholipid. We identified a lead chimeric bispecific antibody with human IgG_4 heavy chain and κ light chain. Subsequent humanization and further engineering of the antibody generated an antibody termed hBS23. Surface plasmon resonance analysis showed that one of the arms of hBS23 recognizes factor IX (FIX) and FIXa, whereas the other arm recognizes FX and FXa (**Supplementary Fig. 1**). Western blotting analysis under reducing conditions showed that the epitope recognized by each arm of hBS23 is located in the FIXa light chain and the FX light chain, respectively (data not shown).

In an enzymatic assay using purified coagulation factors, hBS23 greatly enhanced FX activation, whereas monospecific one-armed antibodies[28] that had either the FIXa- or FX-specific variable region of hBS23 were completely inactive, as was a mixture of the two monospecific one-armed antibodies (**Fig. 2a**). hBS23 did not enhance FX activation in the absence of FIXa (**Fig. 2a**) or phospholipid (**Supplementary Fig. 2**), indicating that hBS23 functions as a cofactor mimicking FVIII and that the reaction required the presence of phospholipid. To compare the mechanistic basis for the cofactor function of hBS23 to that of FVIIIa, we performed a kinetic analysis of FIXa-catalyzed FX activation. Both hBS23 and FVIIIa increased the catalytic rate constant (k_{cat}) and decreased the Michaelis constant (K_m), consequently increasing the catalytic efficiency (k_{cat}/K_m) (**Table 1**). However, the extents to which they each affected k_{cat} and K_m were quite different; compared to FVIIIa, hBS23 showed ten times the effect on decreasing K_m, but 1/140th the effect on increasing k_{cat}, resulting in 1/14th the effect on increasing k_{cat}/K_m.

We next evaluated the activity of hBS23 in human FVIII-deficient plasma. With respect to the time to initiation of clot formation, hBS23 dose-dependently shortened the activated partial thromboplastin time (APTT) in FVIII-deficient plasma both in the presence and absence of inhibitors, whereas the effects of recombinant human FVIII (rhFVIII) on APTT were blocked by the presence of inhibitors (**Fig. 2b**). Doses of hBS23 greater than 30 nM had a greater effect on shortening the APTT compared to 1 U ml^{-1} rhFVIII (100% of normal FVIII activity). The shorter APTT achieved by hBS23 compared to that under normal FVIII activity can be explained by the fact that FVIII requires additional time to be activated by thrombin or FXa, whereas hBS23 does not. To further examine the therapeutic potential of hBS23, we evaluated its effect on thrombin burst in a thrombin generation assay (TGA)[29] in human FVIII-deficient plasma. hBS23 dose-dependently improved thrombin generation parameters, including peak height (defined as the peak of free thrombin concentration) and endogenous thrombin potential (ETP, defined as the area under the curve, a readout of the overall capacity of plasma to form thrombin) even in the presence of inhibitors that rendered rhFVIII completely ineffective (**Fig. 2c–e** and **Supplementary Fig. 3**). With respect to peak height, hBS23 exerted activity equivalent to 0.01 U ml^{-1} (1%) rhFVIII at a concentration of ~30 nM and equivalent to nearly 0.1 U ml^{-1} (10%) rhFVIII at a concentration of ~300 nM. These results suggest that hBS23 has the potential to exert sufficient FVIII-mimetic activity for routine prophylactic use, even in patients with inhibitors who are nonresponsive to FVIII treatment.

To evaluate the *in vivo* hemostatic activity of hBS23, which is highly species specific in the manner in which it exerts FVIII-mimetic activity, we needed a hemophilia A model in nonhuman primates. As nonhuman primates with inherited hemophilia A were unavailable, we established an acquired hemophilia A model. For this purpose, we identified a neutralizing antibody against FVIII from mice, termed VIII-2236, which was cross-reactive to cynomolgus monkey FVIII but not to porcine FVIII (**Supplementary Fig. 4**). Injection of VIII-2236 into cynomolgus monkeys neutralized endogenous FVIII (data not shown) but not exogenous recombinant porcine FVIII (rpoFVIII). We established hemophilia A status by injecting cynomolgus monkeys with VIII-2236 and then artificially induced bleeding (**Fig. 3a**). The monkeys in the control group showed progressive anemia (as assessed by a decrease in hemoglobin levels) and an expanded bruised area (**Fig. 3b,c**). Compared to the control, bolus intravenous administration of 0.3 mg per kg body weight of hBS23 significantly prevented the decrease in hemoglobin levels ($P = 0.0116$) and tended to reduce the bruised area ($P = 0.0522$). This hemostatic activity of hBS23 was comparable to that of twice-daily intravenous administration of 1 U per kg body weight of rpoFVIII (**Fig. 3b,c**), which would

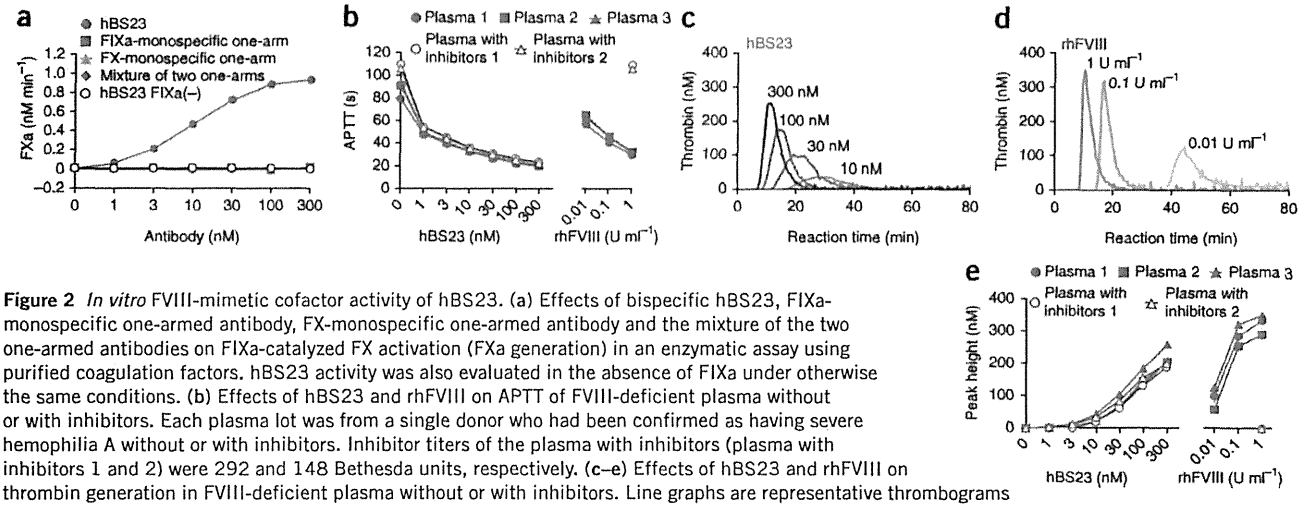

Figure 2 *In vitro* FVIII-mimetic cofactor activity of hBS23. (a) Effects of bispecific hBS23, FIXa-monospecific one-armed antibody, FX-monospecific one-armed antibody and the mixture of the two one-armed antibodies on FIXa-catalyzed FX activation (FXa generation) in an enzymatic assay using purified coagulation factors. hBS23 activity was also evaluated in the absence of FIXa under otherwise the same conditions. (b) Effects of hBS23 and rhFVIII on APTT of FVIII-deficient plasma without or with inhibitors. Each plasma lot was from a single donor who had been confirmed as having severe hemophilia A without or with inhibitors. Inhibitor titers of the plasma with inhibitors (plasma with inhibitors 1 and 2) were 292 and 148 Bethesda units, respectively. (c–e) Effects of hBS23 and rhFVIII on thrombin generation in FVIII-deficient plasma without or with inhibitors. Line graphs are representative thrombograms obtained from FVIII-deficient plasma without inhibitors (plasma 3) in the presence of hBS23 (c) or rhFVIII (d), indicating the time-course of changes in free thrombin concentration during the reaction triggered by factor XIa, phospholipid and Ca^{2+}. Effects of varying concentrations of hBS23 and rhFVIII on a thrombin generation parameter, peak height, is shown for each plasma lot (e). Data were collected in triplicate for each plasma lot and expressed as the means ± s.d., except for the representative thrombograms (in many cases, the bars depicting s.d. are shorter than the height of the symbols).

maintain an rpoFVIII activity of 0.01 U ml^{-1} (1%) or above, according to a simulation of multiple dose injections of rpoFVIII using pharmacokinetic parameters obtained from a single-dose injection study of rpoFVIII in cynomolgus monkeys (A.M., unpublished data). The mean plasma concentration of hBS23 was 5.8 μg ml^{-1} (40 nM) immediately after hBS23 administration and 2.5 μg ml^{-1} (18 nM) at day 3 (the end of the observation period). Neither prothrombin time prolongation nor a decrease in platelet numbers was observed in hBS23-treated monkeys (data not shown), suggesting that hBS23 did not induce consumptive coagulopathy.

We performed a pharmacokinetic study of hBS23 in cynomolgus monkeys by bolus intravenous or subcutaneous injection at a dose of 0.3 mg per kg body weight (**Fig. 3d**). After intravenous injection, hBS23 was cleared from plasma in a biphasic manner (a distribution phase followed by an elimination phase) with a long half-life of 14 d, which is consistent with the general pharmacokinetics of IgG antibodies[27]. The subcutaneous bioavailability of hBS23 was 84%, denoting high subcutaneous bioavailability.

Our data show that a bispecific antibody to FIXa and FX can restore FVIII hemostatic activity by recruiting the two factors into appropriate proximity. We demonstrated that simultaneous recognition of FIXa and FX by a single antibody molecule was required to bring about their activity, as neither a FIXa- or FX-monospecific one-armed antibody, nor a mixture of them, could elicit detectable cofactor activity. Such activity was not detected in the absence of FIXa or phospholipid, indicating that hBS23 exerts its FVIII-mimetic activity only on a tertiary complex consisting of FIXa, FX and phospholipid. Kinetic analysis showed that hBS23 and FVIIIa improved k_{cat} and K_m quite differently: bispecific binding of FIXa and FX by hBS23 improves the interaction between these factors more efficiently than does binding by FVIIIa, but hBS23 has less potential to increase the turnover of the enzyme complex than does FVIIIa. hBS23 was calculated to have 1/14th the catalytic efficiency (k_{cat}/K_m) of FVIIIa. When designing the *in vivo* study, we considered that 0.3 mg per kg body weight of hBS23 would be expected to achieve a plasma concentration that could generate thrombin at a level equivalent to that of 0.01 U ml^{-1} (1%) FVIII *in vitro*. Accordingly, 0.3 mg per kg body weight of hBS23 showed an *in vivo* efficacy comparable to that achieved in a regimen designed to maintain 0.01 U ml^{-1} (1%) or above of FVIII activity, a level sufficient to control hemostasis in patients[3,8]. As the concentration-dependent activity of hBS23 in the TGA in FVIII-neutralized cynomolgus monkey plasma was comparable to that in human FVIII-deficient plasma (**Fig. 2e** and **Supplementary Figs. 3** and **4b,c**), we expect that this *in vivo* efficacy can be translated to the human clinical setting. Although we induced artificial bleeding in this study, the nonhuman primate model that we established has various clinical features of acquired hemophilia, including overt hemorrhage, anemia, bruising and muscle bleeds[30], supporting the extrapolation of this animal study into the clinical setting.

Single-dose pharmacokinetic analysis of hBS23 in cynomolgus monkeys revealed that its half-life (14 d) was slightly longer than that of other humanized or fully human IgG antibodies[31,32]. In addition, whereas the low subcutaneous bioavailability of FVIII necessitates venous access for its injection, hBS23 has high subcutaneous bioavailability (84%) in cynomolgus monkeys. Furthermore, we conducted a simulation study of multiple-dose injections of hBS23 in cynomolgus monkeys using the pharmacokinetic parameters that had been obtained from the pharmacokinetic study. This simulation indicated that once weekly subcutaneous administration of 1 mg per kg body weight of hBS23 would result in a plasma concentration that at any time point would greatly exceed 40 nM (K. Haraya and T. Tachibana, unpublished data), which is the initial plasma concentration in the *in vivo* hemostatic

Table 1 Effects of hBS23 on enzymatic kinetics of FIXa-catalyzed FX activation

Condition	K_m (μM)	V_{max} (nM min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m	Fold increase in k_{cat}/K_m
FIXa	0.0558 ± 0.00281	0.0442 ± 0.00332	0.00111	0.0199	—
+hBS23	0.00253 ± 0.000113	0.996 ± 0.0870	0.996	394	19,800
+FVIIIa	0.0249 ± 0.00247	135 ± 6.08	135	5,420	272,000

The K_m and the maximum velocity (V_{max}) are expressed as the means ± s.d. of three separate experiments. The k_{cat} and the k_{cat}/K_m are calculated from the mean values of K_m and V_{max}. The fold increase in the k_{cat}/K_m by hBS23 or FVIIIa compared to FIXa alone is also shown.

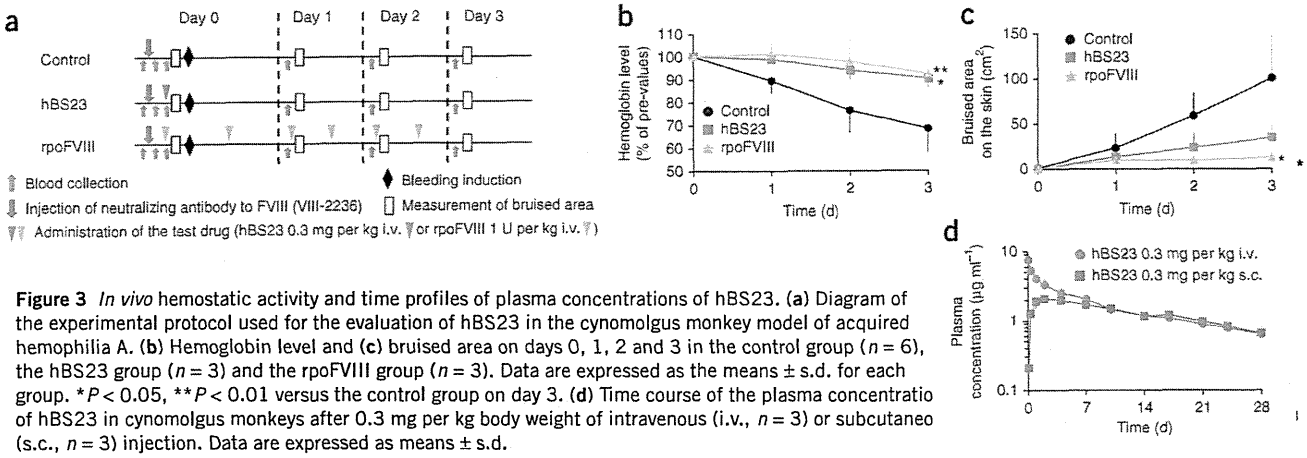

Figure 3 *In vivo* hemostatic activity and time profiles of plasma concentrations of hBS23. (**a**) Diagram of the experimental protocol used for the evaluation of hBS23 in the cynomolgus monkey model of acquired hemophilia A. (**b**) Hemoglobin level and (**c**) bruised area on days 0, 1, 2 and 3 in the control group ($n = 6$), the hBS23 group ($n = 3$) and the rpoFVIII group ($n = 3$). Data are expressed as the means ± s.d. for each group. $*P < 0.05$, $**P < 0.01$ versus the control group on day 3. (**d**) Time course of the plasma concentratio of hBS23 in cynomolgus monkeys after 0.3 mg per kg body weight of intravenous (i.v., $n = 3$) or subcutaneo (s.c., $n = 3$) injection. Data are expressed as means ± s.d.

study. As the half-life of IgG antibodies in humans is generally longer than that in cynomolgus monkeys[33], hBS23 would be expected to have at least a 14-d half-life in humans, which is substantially longer than that of FVIII (0.5 d)[5]. We therefore expect that once weekly dosing of 1 mg per kg body weight of hBS23 would show a continuous hemostatic effect in humans. Effective prophylaxis by once-weekly subcutaneous administration would be an advantageous feature of our bispecific antibody in view of the frequent venous access required for FVIII treatment. Moreover, although maintenance of 1% of normal FVIII activity should be enough for prophylactic use, dosing of hBS23 above 0.3 mg per kg body weight would be expected to exert higher hemostatic activity, as indicated by the dose-dependent activity of hBS23 above 40 nM (the initial plasma concentration of hBS23 after dosing with 0.3 mg per kg body weight in the *in vivo* hemostatic study) in the TGA (at 300 nM, hBS23 had nearly 10% of normal FVIII activity).

Among the alternative therapeutic approaches for hemophilia A that are being pursued, the bispecific antibody described here has some notable advantages. Modified FVIII agents with longer half-lives than the native form of FVIII, including a FVIII Fc fusion protein ($t_{1/2}$ = 18.8 h), are currently being investigated in clinical trials, but although these modified FVIII agents may achieve a reduced injection frequency, they still require venous access[34,35]. Additionally, although gene therapy is in principle a very attractive therapeutic approach that has been actively explored for the past decade, many issues remain to be resolved before it can be used clinically[36].

A notable feature of our bispecific antibody is that it has the potential to overcome the most crucial drawback of treatment using injected FVIII, which would probably also apply to treatment with modified FVIII: the development of inhibitors. Such inhibitors affect not only 30% of the patients with severe disease but also a small percentage of the patients with moderate and mild disease[1,3]. Our experimental results showed that hBS23 activity was not affected by inhibitors. Moreover, considering the generally low immunogenicity of humanized antibodies[26], we expect the incidence of antibodies to hBS23 to be substantially lower than that of antibodies to FVIII. However, as humanized or fully human antibodies are immunogenic in rare cases[26,37], there is still a small risk for the generation of hBS23-specific antibodies in patients. Were hBS23 to become ineffective because of the generation of hBS23-specific antibodies, FVIII treatment could be a viable alternative as long as the hBS23-specific antibodies do not cross-react with FVIII. To estimate the risk of such cross-reactivity, we scanned the sequences of the variable regions of hBS23 to assess sequence similarity to FVIII. This alignment analysis found no similarity between the two molecules (T. Kitazawa, unpublished data); we therefore consider it unlikely that hBS23 treatment would result in the development of hBS23-specific antibodies cross-reactive to FVIII. Thus, we expect that hBS23 treatment would not lead to inhibitor development and could be used in patients who have already developed inhibitors.

In this study, we have generated an agent with the potential to overcome the two major drawbacks of current treatment modalities. Although hBS23 showed efficacy in an animal study, the molecular structure of hBS23 may require further optimization in several ways before the clinical use of such an agent in humans. The first is molecular engineering to enable large-scale manufacturing of the bispecific antibody at clinical grade. Although technological difficulties have prevented any recombinant bispecific antibody from yet reaching the market, various engineering technologies to overcome them have been reported[38,39]. The second is engineering to further reduce the immunogenicity of the humanized antibody. Even though humanized or fully human antibodies generally have low immunogenicity[26], they can be immunogenic if T cell epitopes are present in the complementarity-determining regions[37]. Various approaches to identify and remove such T cell epitopes have been recently reported[26,39]. The third is engineering to prolong the half-life and improve the physicochemical properties of the antibody, which would enable a subcutaneous formulation with an even longer dosing interval[39,40]. The fourth is engineering to further increase the FVIII-mimetic activity of the bispecific antibody. Mutations in the complementarity-determining regions increased the activity of hBS23 over that of the lead chimeric bispecific antibody (data not shown), and the activity of hBS23 could be improved in a same manner. Although hBS23 may require further optimization, this type of bispecific antibody may be able to overcome current limitations and reduce the burden of care for the treatment of hemophilia A.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

T. Kitazawa and T. Igawa led the pharmacological studies and the optimization of the bispecific antibody, respectively, in the program and wrote the manuscript. Z.S. designed the lead chimeric bispecific antibody and hBS23. T. Kojima led the lead identification. H.T. provided ideas on bispecific antibody engineering. T. Suzuki, H.A., T.M., S.I., M.K.-S. and T. Iida generated FVIII-, FIXa- and FX-specific antibodies. T. Soeda, Y.O.-N., A.H., M.F., C.M., E.T., T. Toyoda and A.U. performed the *in vitro* experiments. K.E. and S.S. performed the affinity analyses. Y. Kikuchi, T.W., M.W. and M.G. purified the bispecific antibody and the coagulation factor. A.M. and K.Y. performed the *in vivo* pharmacological study. K. Haraya and T. Tachibana performed the pharmacokinetic study. H.S. and Y. Kawabe provided direction and guidance for the various functional areas. M.S. and A.Y. provided advice on the program from the viewpoints of their medical expertise in hemophilia. K. Hattori provided the hypothesis and directed and organized the program.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

The care and use of laboratory animals. Cynomolgus monkey, mouse, rat and rabbit studies were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Chugai Pharmaceutical under the approval of the company's Institutional Animal Care and Use Committee and also in compliance with the "Act on Welfare and Management of Animals" in Japan. The company is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, a nonprofit organization that promotes the humane treatment of animals in science through voluntary accreditation and assessment programs (http://www.aaalac.org/). We conducted the cynomolgus monkey research adhering to the principles stated in the US National Research Council's Guide for the Care and Use of Laboratory Animals.

Generation of hBS23, a humanized bispecific antibody to FIXa and FX. We prepared the genes of the variable regions of FIXa- and FX-specific antibodies from 46 female mice, 34 female rats and 12 female rabbits that had been immunized with human FIXa or FX (Enzyme Research Laboratories) and inserted the genes into an expression vector containing the constant region of human IgG. The constant region of all the expressed bispecific antibodies had a S238P (EU numbering) substitution, intended to improve hinge stabilization[41], and 'knobs-into-holes' substitutions, intended to increase the desired bispecific combination by promoting heterodimerization efficiency of the two heavy chains[38]. To express a series of bispecific antibodies in HEK 293 cells, we co-transfected a mixture of four expression vectors encoding the heavy and light chains of FIXa- and FX-specific antibodies. After culturing the transfectants, we individually collected and incubated each of the culture supernatants for 2 h with 10 nM FIXa, 10 nM FX and 62.5 μM synthetic phospholipid in 50 mM Tris-buffered saline (TBS) containing 5 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.6). The phospholipid consisted of 10% phosphatidylserine, 60% phosphatidylcholine and 30% phosphatidylethanolamine (Avanti Polar Lipids) and was prepared as previously described[42]. After stopping the reaction by the addition of EDTA, we added a chromogenic substrate specific to FXa (S-2222, Chromogenix) and evaluated the ability of each culture supernatant to promote FXa generation by measuring the absorbance at 405 nm. From approximately 40,000 bispecific combinations, we selected a lead chimeric bispecific antibody based on its ability to generate FXa and the feasibility of using only one light chain, the so-called common light chain. The combination of two heavy chains and two light chains results in ten possible combinations, of which only one yields the desired bispecific antibody; however, use of a common light chain for the two arms of the bispecific antibody reduces the number of combinations of heavy and light chains to three[38]. To generate hBS23, we further humanized the lead chimeric bispecific antibody by complementarity-determining region grafting[43] and engineered it to have stronger FVIII-mimetic activity and better pharmaceutical properties. We transiently expressed the one light chain and two heavy chains of hBS23 in HEK 293 cells and purified it with recombinant Protein A and gel permeation chromatography. We also prepared in the same way one-armed monospecific antibodies[28], each of which have one arm containing either the FIXa- or the FX-specific variable region of hBS23, as well as two-armed monospecific antibodies, in which both arms contain the FIXa- or FX-specific variable region of hBS23.

Characterization of antigen-antibody interaction of hBS23 using surface plasmon resonance. We analyzed the interactions of FIX, FIXa, FX and FXa with the corresponding variable regions of hBS23 by surface plasmon resonance using a Biacore T200 system (GE Healthcare). First, we immobilized MabSelect SuRe Ligand (recombinant Protein A; GE Healthcare) onto a CM4 sensor chip (GE Healthcare) that had been preactivated with NHS/EDC and predeactivated with ethanolamine (Amine Coupling Kit; GE Healthcare). To capture the test antibodies on the sensor chip, we injected each of the FIXa- or the FX-monospecific two-armed IgG antibodies into flow cell 2 and natalizumab (Biogen-Idec) as control human IgG_4 antibody into flow cell 1. We next injected each analyte (0 as baseline, 80, 160, 320, 640, or 1,280 nM human FIX, FIXa, FX, or FXa), which had been dissolved in running buffer (10 mM HEPES, 150 mM NaCl, 0.05% (vol/vol) Surfactant P20, 2.5 mM $CaCl_2$ (pH 7.4); GE Healthcare), into both flow cells on the sensor surface at a flow rate of 30 μl min^{-1} to monitor the association phase for 120 s and the dissociation phase for 30 s. The data were analyzed by the 1:1 binding model in the Biacore T200 Evaluation software (version 1.0, GE Healthcare).

Enzymatic assays. We evaluated the conversion rate of FX to FXa in an enzymatic assay using purified coagulation factors. The assay system consisted of 1 nM human FIXa, 140 nM human FX, 20 μM phospholipid and either hBS23, FIXa- or FX-monospecific one-armed antibodies, or a mixture of two monospecific one-armed antibodies, and measured FXa generation at room temperature for 2 min in TBS containing 5 mM $CaCl_2$ and 0.1% (wt/vol) BSA. We also performed the experiments in the absence of FIXa or phospholipid under otherwise the same conditions. We stopped the reaction by adding EDTA at various time points. After adding S-2222 chromogenic substrate, we measured absorbance at 405 nm to determine the rate of FXa generation and quantified it by extrapolation from a standard curve prepared using known amounts of human FXa (Enzyme Research Laboratories). Data were collected in triplicate.

Kinetic analyses. We monitored the rate of FIXa-catalyzed FX activation by incubating 1 nM human FIXa, various concentrations of human FX and 20 μM phospholipid with either 300 nM hBS23 or 30 U ml^{-1} rhFVIII (Bayer Healthcare) that had been preactivated by thrombin and converted into FVIIIa in TBS containing 5 mM $CaCl_2$ and 0.1% (wt/vol) BSA (pH 7.6) at room temperature. We also performed the experiments in the absence of hBS23 and FVIIIa under the same conditions, except that we altered the FIXa concentration to 40 nM. We stopped the FXa generation reaction by adding EDTA at various time points. The rates of FXa generation were determined in the same way as described in the previous section. The values of K_m and V_{max} were calculated using a nonlinear regression data analysis program (GraphPad PRISM version 5.04).

Preparation of rpoFVIII. We expressed a B domain–deleted form of rpoFVIII in Chinese hamster ovary cells by stable transfection. The vector employed for the transfection was designed on the basis of a previously reported sequence of the *F8* (*Sus scrofa*) gene (GenBank: NM_214167) and the sequence of B domain–deleted human FVIII (refs. 44,45). After diluting the supernatant of rpoFVIII-containing medium with 20 mM Tris-HCl and 5 mM $CaCl_2$ (pH 7.6), we loaded it onto a Q Sepharose Fast Flow column (GE Healthcare) equilibrated with the same buffer. We then eluted rpoFVIII with a linear 0 to 1 M NaCl gradient in the same buffer. We pooled and concentrated the fractions containing rpoFVIII and then subjected them to gel permeation chromatography on a HiLoad 26/60 Superdex200pg column (GE Healthcare) equilibrated in 150 mM NaCl, 10 mM histidine-HCl, 5 mM $CaCl_2$, 0.1% (wt/vol) sucrose and 0.02% (wt/vol) polysorbate 80 (Junsei Chemical) (pH 7.0). We again pooled the fractions containing rpoFVIII and aseptically filtered the mixture. We assessed the FVIII activity (U ml^{-1}) of the purified rpoFVIII in a one-stage coagulation assay, one of the standard assays for determining FVIII activity, using normal human plasma (Siemens Healthcare) as the standard.

Generation of VIII-2236, a neutralizing antibody against FVIII. We established hybridomas producing an antibody against human FVIII from eight female mice that had been immunized with rhFVIII. Using APTT measurements and the TGA, we identified VIII-2236 as a neutralizing antibody against FVIII with cross-reactivity to cynomolgus monkey FVIII but not to porcine FVIII.

APTT measurements. We measured APTT with standard techniques using Thrombocheck APTT-SLA (Sysmex). We employed three lots of commercially available human FVIII-deficient plasma without inhibitors (<1% normal FVIII activity) and two lots with inhibitors (George King Bio-Medical). Each lot contained frozen fresh plasma from a single donor with severe hemophilia A. We also used the pooled citrated plasma of nine male cynomolgus monkeys, which contained 300 μg ml^{-1} VIII-2236 (2.6% by volume for VIII-2236 solution). Data were collected in triplicate.

Thrombin generation assays. We obtained thrombograms by calibrated automated thrombography[46] using a 96-well plate fluorometer (Thermo Fisher

Scientific Instruments) equipped with a 390/460 filter set, a dispenser and analyzing software (Thrombinoscope software version 3.0.0.29, Thrombinoscope BV). Briefly, into each well we dispensed 80 µl of a plasma solution containing hBS23, rhFVIII or rpoFVIII added to the human FVIII-deficient plasma or the FVIII-neutralized pooled cynomolgus monkey plasma (<1% by volume). We then added 20 µl of the triggering solution containing 0.47 nM human factor XIa (Enzyme Research Laboratories) and 20 µM phospholipid but no Ca^{2+}. For calibration, we added 20 µl of Thrombin Calibrator (Thrombinoscope BV) instead of the triggering solution. To initiate the reaction, 20 µl of FluCa reagent prepared from FluCa kit (Thrombinoscope BV) was dispensed by the instrument as programmed. The instrument's software analyzed the thrombograms, peak height and ETP. Data were collected in triplicate.

***In vivo* experiments in the acquired hemophilia A model.** We used 12 male cynomolgus monkeys (2.50–3.60 kg and 3–4 years of age), housed individually, which were divided into three groups, receiving hBS23 (n = 3), rpoFVIII (n = 3) or neither agent (control group; n = 6). The experimental protocol is diagrammed in **Figure 3a**. On day 0, each monkey intravenously received an adjusted dose of VIII-2236 that would result in an APTT twice as long as the baseline APTT for that monkey. The means ± s.d. of the adjusted VIII-2236 dose was 3.6 ± 3.8 mg per kg body weight in the control group, 3.3 ± 2.3 mg per kg body weight in the hBS23 group and 4.5 ± 1.9 mg per kg body weight in the rpoFVIII group. Two hours thereafter, we anesthetized each monkey by isoflurane inhalation and measured the area of purple-colored bruising on the skin of the entire body of monkeys whose pelage had been closely cropped. We next intravenously administered the test drug to each monkey in the hBS23 or the rpoFVIII group at 0.3 mg per kg or 1 U per kg body weight, respectively. We then induced bleeding in the monkeys by performing an injury procedure: 1-cm-deep insertions of an 18-G needle into muscles at 16 sites (four sites in each thigh, two sites in each upper arm and two sites in each forearm). We maintained the anesthesia until the completion of this injury procedure. In our previous experience, more severe injury procedures did not decrease hemoglobin levels in normal monkeys, so we did not expect this procedure to decrease hemoglobin in the absence of FVIII neutralization. On days 1, 2 and 3, we again anesthetized the monkeys and measured the bruised area. After completing the evaluation of the bruised area on day 3, the monkeys were killed humanely. To each of the monkeys in the rpoFVIII group, we administered additional intravenous injections of rpoFVIII (1 U per kg body weight) in the mornings of days 1 and 2 just before bruise measurement and also in the evenings of days 0, 1 and 2 (twice a day, total of six administrations of rpoFVIII). We collected blood samples before and after injecting VIII-2236, after the first administration of the test drug on day 0 and before measuring the bruised area on days 1, 2 and 3 to confirm the neutralizing effect of VIII-2236 against endogenous FVIII and to determine the concentration of hBS23 and the hemoglobin level. We expressed the change of hemoglobin level as a percentage of the value on day 0 (after the injection of VIII-2236) for the respective monkey.

Pharmacokinetic study. We intravenously or subcutaneously administered a single dose of 0.3 mg per kg body weight of hBS23 to male cynomolgus monkeys (2.57–3.10 kg and 2–3 years of age, n = 3 for the intravenous administration group and n = 3 for the subcutaneous administration group). We collected blood samples before the administration and 0.5 h (intravenous administration only), 2 h (subcutaneous administration only), 8 h, 1 d, 2 d, 4 d, 7 d, 10 d, 14 d, 17 d, 21 d, 24 d and 28 d after the administration. Pharmacokinetic parameters were calculated by WinNonlin Professional software (version 4.0.1, Pharsight). The subcutaneous bioavailability was calculated by dividing the AUC_{inf} for the subcutaneous administration by the AUC_{inf} for intravenous administration.

Statistical analyses. Data are presented as means ± s.d. In the *in vivo* experiments employing the acquired hemophilia A model, significant differences in the data of day 3 were determined by the parametric Dunnett's multiple comparison test (two-tailed) compared to the control group (SAS preclinical package version 5.00, SAS Institute Japan).

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Four weeks into his admission he developed sudden-onset macroscopic haematuria with a significant anaemia requiring packed red blood cells. A renal biopsy was planned, as a surgical cause was not identified and a preprocedure coagulation check revealed an abnormally prolonged aPTT of 87 s (reference range: 22–33 s). Subsequent mixing studies and factor assays confirmed a factor VIII inhibitor with a titre of 30 BU and a factor VIIIc activity of <1 IU dL^{-1}. There were no other clinically significant foci of bleeding at that stage. Due to concerns with ongoing infection, haematuria and potential for further bleeding, he received a trial of high-dose intravenous immunoglobulin at a dose of 1 g kg^{-1} for 2 days, which was not successful in correcting his coagulopathy. He was continued to be supported with packed red cell transfusion (23 units in total) and activated FVII at a dose of 90 μg kg^{-1} (37 mg total) as bolus doses given at six hourly intervals for a total of six doses for his ongoing blood loss anaemia; rVIIa was ceased after a total of six doses as there had been no reduction in macroscopic haematuria. To exclude a drug-induced inhibitor, ceftazidime was changed to meropenem without improvement.

In view of comorbidities and concerns about using steroid-based regimens as first-line immunosuppression for acquired haemophilia A, a decision was made to treat with Rituximab (Roche Products Pty Limited, Dee Why, Australia) at a dose of 375 mg m^{-2} IVI q weekly for 4 weeks, as described previously [5]. He was given lamivudine as hepatitis B prophylaxis due to known carrier status. Transfusion requirements settled into the fourth week of Rituximab therapy and the aPTT improved to 46 s. His FVIIIc activity has improved to 95 IU dL^{-1} with a corresponding decrease of inhibitor to 0.5 BU 4 months from the start of therapy.

The renal function returned to his impaired baseline of 250 mmol L^{-1} and the patient has been stable with hemoglobin of 115 g L^{-1}. His melioidosis infection continues to be treated with doxycyline and there has been no flare of infection to date.

The cause of the acquired inhibitor in our patient is uncertain, the possibilities include the underlying infection, cephalosporin therapy and his age. Acquired inhibitors to factor VIII have also been well described in a number of conditions including post-partum state and malignancy; however, to the best of our understanding there are few case reports in association with therapy of infectious agents [6], with no previous description of an association with melioidosis. Intravenous immunoglobulin has an immunomodulating effect in autoimmune disorders [7], and it could have had a synergistic effect on the Rituximab infused 2-weeks later. This case also highlights the utility of Rituximab in the treatment of acquired haemophilia A in situations where corticosteroids or other immunosuppressive agents cannot be instituted. Rituximab would also be an excellent choice when compliance with oral agents is an issue in patients living remotely from supervised medical care. The authors do acknowledge that significant immunosuppression or adverse events can be experienced after receiving Rituximab and vigilance has to be exercised.

Author's contribution

AK prepared the manuscript; FS and BC reviewed the manuscript.

Disclosures

Akash Kalro has received travel honoraria for a national meeting from Roche®. All other authors have declared no conflicting interests.

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Complete immunotolerance induction after FEIBA prophylaxis in a haemophilia A patient with high-titre inhibitor

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The standard goal in the current management of haemophilia children is the early establishment of prophylaxis using recombinant factor VIII or IX (FVIII/IX) agents. The development of inhibitors neutralizing the clotting factor activity is the most challenging complication associated with the treatment. Immune tolerance induction (ITI) is usually applicable for haemophilia patients having low, but not extremely high-titre of FVIII or FIX inhibitor [1]. Bypass therapy using recombinant activated FVII (rFVIIa) (Novoseven®) or activated prothrombin complex concentrate (aPCC) (anti-inhibitor coagulant complex, FEIBA VH®) is the only measure to control bleeding in

Table 1. Major bleedings and anamnestic responses triggered by high-dose FVIII infusions during prolonged bypass therapy.

Years of age	Bleeding episodes and events	Titre of FVIII inhibitor (BU mL^{-1})	Managements
Birth	Hepatic bleeding		High-dose FVIII conc.
1	Intracranial haemorrhage (first)	0.9*	High-dose FVIII conc. On-demand FVIII conc.
2	Intracranial haemorrhage (second)		High-dose FVIII conc. On-demand FVIII conc.
7	High titre inhibitor detected	127	On-demand PCC
10	Intracranial haemorrhage (third)	1 → 989	High-dose FVIII conc. Regular PCC
14	Quadriceps bleeding, shock	8 → 4	rFVIIa, high-dose PCC Regular PCC, aPCC
19	Left knee joint bleeding	<0.6* → 1	High-dose FVIII conc. Regular aPCC
19	ITI	1 → 3 → <0.3**	High-dose FVIII conc. to recombinant FVIII agent

PCC, prothrombin complex concentrate; aPCC, activated prothrombin complex concentrate; ITI, immune tolerance induction; FVIII, human coagulation factor VIII; conc., concentrate; rFVIIa, recombinant activated factor VII.
The minimum detectable levels of inhibitor are 1, 0.6 (*) and 0.3 (**) BU mL^{-1} respectively.

patients with high-titre inhibitors. Recent retrospective studies indicated the safety and efficacy of aPCC or rFVIIa in decreasing the frequency of bleeding episodes in patients with high-titre inhibitors. However, anamnestic response is one of the major obstacles for the introduction of ITI, because it often occurs even after aPCC infusion in patients with haemophilia A or B and each high responding inhibitor. rFVIIa agent does not precipitate anamnesis, although the shorter biological half-life confronts a dilemma for the effective prophylaxis. Thus, the ultimate goal, 'the eradication of high responding inhibitor in hemophilia children' is beyond our scope of practice. We herein report a paediatric case of haemophilia A with high-titre inhibitor who succeeded in ITI until age 20 years following 4 years' FEIBA prophylaxis.

A 19-year-old Japanese boy with haemophilia A was hospitalized because of the left knee joint pain. The patient had a high-titre FVIII inhibitor, and the bleedings were successfully controlled with self-infusion of FEIBA. The detailed course was reported previously [2]. The diagnosis of haemophilia A was based on a neonatal hepatic bleeding and <1% of FVIII activity. For 7 years' on-demand therapy with plasma-derived FVIII concentrates (Hemofil-M®/Crosseight-M®), intracranial bleedings adjacent to a subarachnoid cyst repeated at age 1 and 2 year(s). Thereafter, he showed 127 BU/mL of FVIII inhibitor (Table 1). Despite the on-demand therapy with Proplex-ST®, he frequently bled into the target joints. At age 10 years, 1 BU/mL of the inhibitor level elevated to 989 BU/mL after high-dose Crosseight-M therapy for the third intracranial haemorrhage. During the consequent regular infusions of Proplex (50 U/kg/day, tri-weekly), joint and mucosal bleedings recurred. An accidental quadriceps bleeding and shock at 14 years of age prompted FEIBA prophylaxis (50 U/kg/day, tri-weekly), that led to a successful school life followed by a professional life of care-worker in hospital. He was on dietary therapy for type 2 diabetes mellitus since age 15 years. No adverse events occurred during the self-FEIBA infusion without the use of central venous access devices.

At age 19 years, he visited our emergency room because of the left knee injury resulting from an accidental fall from stairs on collision with passengers at the station. On admission, he complained of severe knee joint pain, but the vital signs were unremarkable. Weight and height were 72 kg and 163 cm (BMI 27.1) respectively. There was no anaemia, jaundice, skin abrasion or bone fracture. Cardiopulmonary sounds were normal. Flat abdomen showed no hepatosplenomegaly. Neurological findings were normal. The swollen left knee was hot and tender with the restriction of movement. No other joint disease was found assessed by physical function. An echography of the affected knee indicated bleedings into the joint space but not adjacent muscles. Peripheral blood counts showed leukocyte of 14.51 × 10^9/L, with 87% neutrophils, 9% lymphocytes and 4% monocytes, red cell counts of 4,830 × 10^9/L, haemoglobin of 15.1 g/dL, haematocrit of 43.3% and a platelet count of 218 × 10^9/L. Blood glucose level was 162 mg/mL and haemoglobin A1c was 7.7%. Blood chemistries showed normal liver and kidney functions. Lipid profile and creatinine kinase levels were normal. C-reactive protein concentration was 0.13 mg/dL. There was no evidence of infection. Coagulation studies were as follows: prothrombin time 10.9 s (control: 11.2 sec), activated partial thromboplastin time 102.1 s (control: 34.1 sec), fibrinogen 293 mg/dL (reference range [rr]: 150-400 mg/dL) and fibrinogen/fibrin degradation product 6.4 μg/mL (rr: 0–5.0 μg/mL). Coagulation factor assays revealed <1% FVIII level and <0.6 BU/mL FVIII inhibitor assessed by the high-sensitivity method.

During the immediate infusions of rFVIIa (4.8 mg/dose, twice) and arthrocentesis (aspirated volume: 20 mL), the joint symptoms were exacerbated. Crosseight-M therapy (6000 U [42 U/kg]/day) led to a drastic improvement. FVIII inhibitor increased to 1.0 BU/mL on 10 days after the start of Crosseight-M, when the daily dose was tapered to the half (3000 U/day). The FVIII activity was 32% after 6000 U infusion of the agent. Re-escalated dose (6000 U) of Crosseight-M continued for 7 days with the exercise for rehabilitation. There remained no gait disturbance, although the range of motion of the affected knee was limited to 130°. After hospital discharge, alternate-day regimen of 5000U Crosseight-M was commenced as ITI. FVIII inhibitor peaked at 3 BU/mL 8 months after discharge, although he often skipped the infusions. During the period of ITI using Crosseight-M (5000 U [50mL]/alternate day), substantial consumption of the agent was less than half of the predicted doses. Then, a recombinant agent was introduced for ITI (Advate® 4000 U [20mL]/day, tri-weekly). The volume and regimen were tolerated, and induced no anamnestic response. FVIII recovery and half-life increased >60% and >6 h respectively. FVIII inhibitor level decreased to <0.3 BU/mL. Enzyme-linked immunoassay revealed undetectable FVIII inhibitor in immunoglobulin (Ig)G, IgM or any IgG subclass assessed by the established method [3].

Haemophilia patients having >200 BU/mL of inhibitor titre often fail to eradicate the antibody against FVIII even if tried ITI. The International Consensus Panel noted that for poor risk ITI patients, defined by a historical titre of >200 BU/mL and/or a pre-ITI titre of >10 BU/mL and/or an interval of >5 years as inhibitor diagnosis, efficacy data is limited to high-dosing regimens (>200 U/kg/day) [1]. The present patient fulfilled all the poor risk criteria and repeated cerebral bleedings. In such patients, FVIII exposure and anamnestic bypass therapy should be avoided for the prevention and treatment of bleeding during the deferral period to ITI. Our patient showed no significant anamnestic response during the administration of PCC and aPCC, and then received the low-dose FEIBA-prophylaxis. The major concern is the optimal time of ITI induction in patients with haemophilia A

inhibitor whose inhibitor titre declined during the regular bypass therapy. ITI could be started at 5-10 BU/mL if the titre does not decline over a period of 1-2 years and/or if inhibitor development or persistence is associated with severe or life-threatening bleeding [1]. During the FEIBA prophylaxis in our patient, the inhibitor titre came to undetectable levels (<0.6 BU/mL). However, subsequent ITI provoked a mild anamnestic response to 3 BU/mL. Watanabe et al. [4] described a 11-year-old boy with severe haemophilia B and high responding anti-FIX inhibitor with a historical peak of 70 BU/mL, the titre of which gradually decreased more than 2 years after the regular infusions of FEIBA. The total amount of FIX in aPCC might contribute to the successful ITI in the patient with haemophilia B. On the other hand, FEIBA contains only a small amount of FVIII fragments [5]. Although the changing time from FEIBA prophylaxis to the start of ITI is hard to determine, the regular challenge of FVIII fragments might need prolonged time to result in the desensitization.

There have been a growing number of reports on the aPCC or rFVIIa prophylaxis for haemophilia patients with high-titre inhibitor [6,7], although both agents share the expense hurdle. FEIBA prophylaxis might have a benefit for preventing life-threatening bleedings and reducing the frequency of bleeding episodes. Not all patients documented the increase of FVIII inhibitor titres. The reported adverse events during the prophylaxis included catheter port infections and/or sepsis, decreased fibrinogen levels, peripheral thrombophlebitis, but no thrombotic complications. During 4 years' self-FEIBA prophylaxis (50 U/kg/day, bi-/tri-weekly), the obese boy experienced no adverse events in the active daily life. Recently, Valentino et al. [8] conducted the meta-analysis for 34 patients (including one haemophilia B) with mean historical peak of inhibitor 1267 BU/mL, who started prophylaxis at mean age of 10.1 years and continued the management for at most 6 years. During the regimen (FEIBA 50-100 U/kg, 3-4 times per week), there was 64% reduction in bleeding episodes. Some patients had no impact of anamnesis on the prophylactic efficacy. No one had thrombotic or other complications. The present patient attained the complete tolerance within 6 months from the peak anamnesis of 3 BU/mL by low-dose ITI regimen of recombinant FVIII (50 U/kg/day, tri-weekly). Both the dose and the time to success of ITI were as expected [9]. Successful ITI occurs without the deletion of FVIII-specific T-cells. The favourable effects might arise from the potential inhibition of danger signals that stimulate $CD4^+$ T-cell dependent antibody production [10]. Taken together, FEIBA prophylaxis may be effective and safe in both ITI and non-ITI settings, unless the inhibitor titre rises during the prophylaxis. Primary and secondary rFVIIa prophylaxis could also reduce the bleeding episodes in patients with haemophilia A and inhibitor. Further prospective studies are needed to optimize the regimen of bypassing agents for prophylaxis aiming at the subsequent ITI.

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Contributions to authorship

Nanishi E, Doi T and Ohga S treated the patient, and wrote the manuscript. Ishimura M, Ihara K, Takada H supported home-infusion therapy. Shima M measured the inhibitor assessed by the high sensitivity methods and enzyme-linked immunoassay. Hara T organized the clinical management and supported to complete the manuscript.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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A putative inhibitory mechanism in the tenase complex responsible for loss of coagulation function in acquired haemophilia A patients with anti-C2 autoantibodies

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Summary

Acquired haemophilia A (AHA) is caused by the development of factor (F)VIII autoantibodies, demonstrating type 1 or type 2 inhibitory behaviour, and results in more serious haemorrhagic symptoms than in congenital severe HA. The reason(s) for this remains unknown, however. The global coagulation assays, thrombin generation tests and clot waveform analysis, demonstrated that coagulation parameters in patients with AHA-type 2 inhibitor were more significantly depressed than those in patients with moderate HA with similar FVIII activities. Thrombin and intrinsic FXa generation tests were significantly depressed in AHA-type 1 and AHA-type 2 compared to severe HA, and more defective in AHA-type 1 than in AHA-type 2. To investigate these inhibitory mechanism(s), anti-FVIII autoantibodies were purified from AHA plasmas. AHA-type 1 autoantibodies, containing an anti-C2 ESH4-epitope, blocked FVIII(a)-phospholipid binding, whilst AHA-type 2, containing an anti-C2 ESH8-epitope, inhibited thrombin-catalysed FVIII activation. The coagulation function in a reconstituted AHA-model containing exogenous ESH4 or ESH8 was more abnormal than in severe HA. The addition of anti-FIX antibody to FVIII-deficient plasma resulted in lower coagulation function than its absence. These results support the concept that global coagulation might be more suppressed in AHA than in severe HA due to the inhibition of FIXa-dependent FX activation by steric hindrance in the presence of FVIII-anti-C2 autoantibodies. Additionally, AHA-type 1 inhibitors prevented FVIIIa-phospholipid binding, essential for the tenase complex, whilst AHA-type 2 antibodies decreased FXa generation by inhibiting thrombin-catalysed FVIII activation. These two distinct mechanisms might, in part, contribute to and exacerbate the serious haemorrhagic symptoms in AHA.

Keywords

Acquired haemophilia A, anti-C2 autoantibody, thrombin generation, tenase complex, FXa generation

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Introduction

Factor (F)VIII, a protein deficient or defective in individuals with severe congenital bleeding disorder, haemophilia A (HA), functions as a cofactor in the tenase complex, responsible for phospholipid (PL)-dependent conversion of FX to FXa by FIXa (1). FVIII circulates as a complex with von Willebrand factor (VWF) that protects and stabilises the cofactor (2). 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, generating a heavy chain (HCh) consisting of A1 and A2 domains together with heterogenous fragments of partially proteolysed B domain linked to a light chain (LCh) consisting of A3, C1, and C2 domains (3). The catalytic efficiency of FVIII in the tenase complex is markedly enhanced by conversion into FVIIIa, by limited proteolysis by thrombin (and FXa) (4). Both enzymes proteolyse the HCh at Arg^{372} and Arg^{740}, and produce 50-kDa A1 and 40-kDa A2 subunits. The 80-kDa LCh is cleaved at Arg^{1689} generating a 70-kDa subunit. Proteolysis at Arg^{372} and Arg^{1689} is essential for generating FVIIIa cofactor activity (5). FVIIIa activity is down-regulated by serine proteases including activated protein C, following cleavage at Arg^{336} (4, 6).

FVIII inhibitors develop as alloantibodies (alloAbs) in severe HA patients multi-treated with FVIII concentrates, and also as autoantibodies (autoAbs) in previously normal individuals, particularly in elderly people, patients with autoimmune diseases, pregnant women, and women in the postpartum period. The appearance of autoAbs usually results in severe haemorrhagic symptoms in what is described as acquired HA (AHA). Antibodies of this nature inhibit FVIII activity (FVIII:C) either completely or incompletely at saturating concentrations, corresponding to type 1

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/

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.

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.

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 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

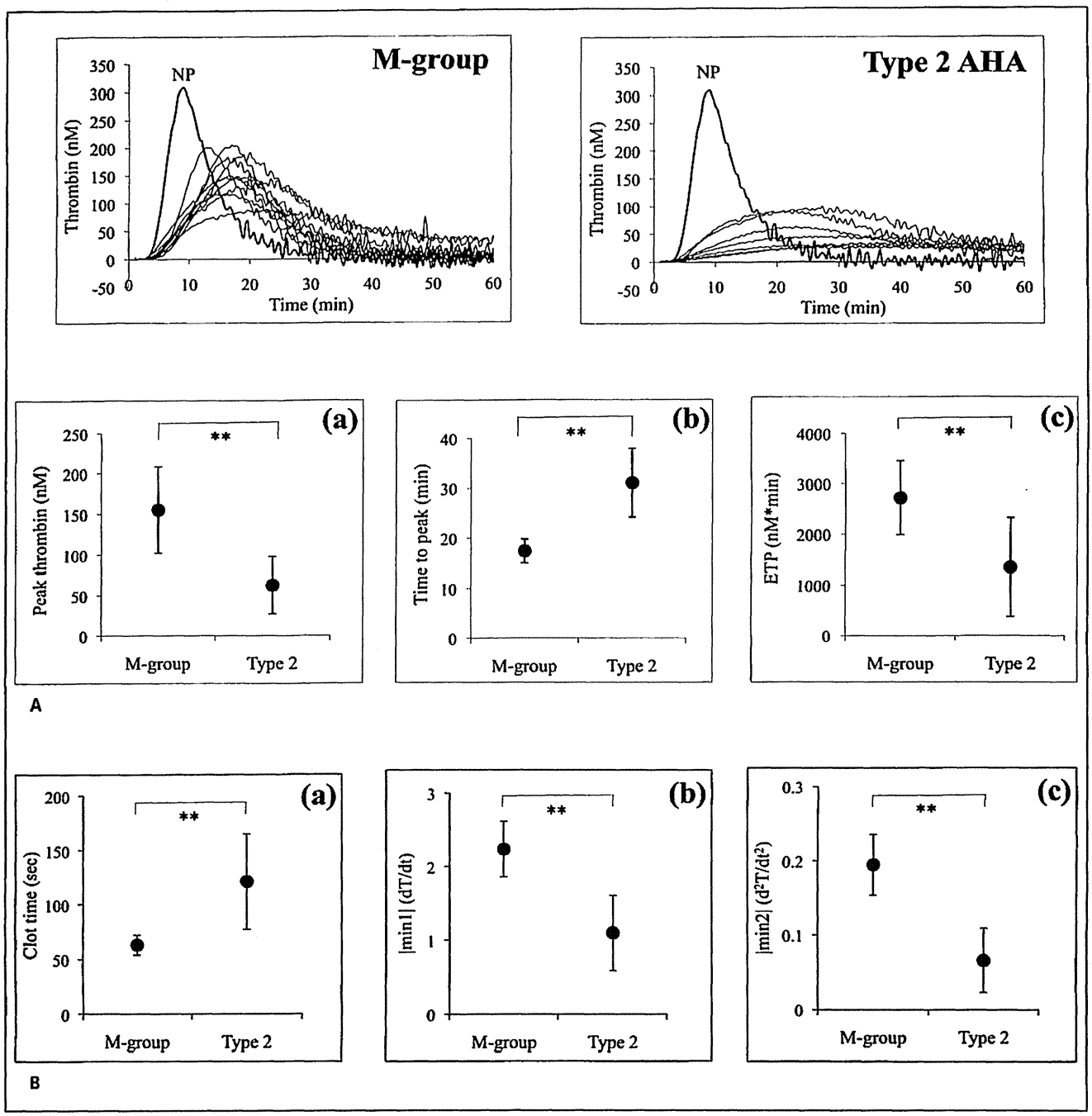

Figure 1: TGT and clot waveform analysis on patient's plasmas in the M-group and type 2 AHA. A) TGT-assay; upper panels: Plasma samples obtained from the M-group patients and type 2 AHA were preincubated with TF (0.5 pM), PL (4 μM) and ellagic acid (0.3 μM), followed by the addition of $CaCl_2$. Thrombin 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.