kinetics in blood from patients with hemophilia A. *Blood Coagul Fibrinolysis* 2010; **21**: 539–46.

10 Allen GA, Persson E, Campbell RA, Ezban M, Hedner U, Wolberg AS. A variant of recombinant factor VIIa with enhanced procoagulant and antifibrinolytic activities in an in vitro model of hemophilia. *Arterioscler Thromb Vasc Biol* 2007; **27**: 683–9.

11 Sørensen B, Persson E, Ingerslev J. Factor VIIa analogue (V158D/E296V/M298Q-FVIIa) normalises clot formation in whole blood from patients with severe haemophilia A. *Br J Haematol* 2007; **137**: 158–65.

12 Tranholm M, Kristensen K, Kristensen AT, Pyke C, Røjkjær R, Persson E. Improved hemostasis with superactive analogs of factor VIIa in a mouse model of hemophilia A. *Blood* 2003; **102**: 3615–20.

13 Holmberg HL, Lauritzen B, Tranholm M, Ezban M. Faster onset of effect and greater efficacy of NN1731 compared with rFVIIa, aPCC and FVIII in tail bleeding in hemophilic mice. *J Thromb Haemost* 2009; **7**: 1517–22.

14 Møss J, Scharling B, Ezban M, Møller Sørensen T. Evaluation of the safety and pharmacokinetics of a fast-acting recombinant FVIIa analogue, NN1731, in healthy male subjects. *J Thromb Haemost* 2009; **7**: 299–305.

15 World Medical Association. Declaration of Helsinki. Ethical Principles for Medical Research Involving Human Subjects. 52nd WMA General Assembly, Edinburgh, Scotland, October 2000. Last amended with Note of Clarification on Paragraph 29 by the WMA General Assembly, Washington 2002, and Note of Clarification on Paragraph 30 by the WMA General assembly, Tokyo 2004.

16 International Conference on Harmonisation. ICH Harmonised Tripartite Guideline. Good Clinical Practice. 01-May-1996.

17 Ministry of Health and Welfare Ordinance on Good Clinical Practice. MHW Ordinance No. 28; 27 March 1997) and revisions thereof (J-GCP).

18 Morrissey JH, Macik BG, Neuenschwander PF, Comp PC. Quantitation of activated FVII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. *Blood* 1993; **81**: 734–44.

19 Petersen LC, Karpf DM, Agersø H, Hermit MB, Pelzer H, Persson E, Nichols TC, Ezban M. Intravascular inhibition of factor VIIa and the analogue NN1731 by antithrombin. *Br J Haematol* 2011; **152**: 99–107.

20 Agersø H, Brophy DF, Pelzer H, Martin EJ, Carr M, Hedner U, Ezban M. Recombinant human factor VIIa (rFVIIa) cleared principally by antithrombin following intravenous administration in hemophilia patients. *J Thromb Haemost* 2011; **9**: 333–8.

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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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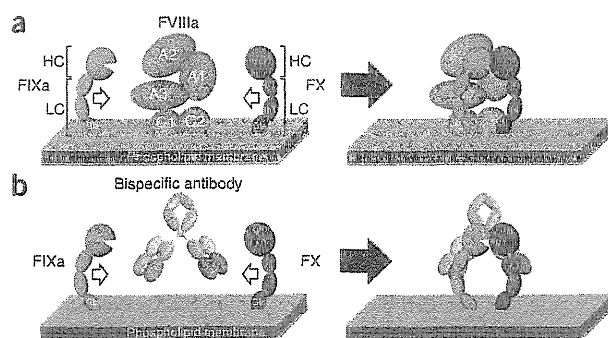

Figure 1 Schematic illustrations of the action of FVIIIa or of a bispecific antibody as a cofactor promoting the interaction between FIXa and FX. (**a**) FVIIIa consists of the A1 subunit, the A2 subunit and the light chain (A3, C1 and C2 subunits). FVIIIa forms a complex with FIXa and supports the interaction 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

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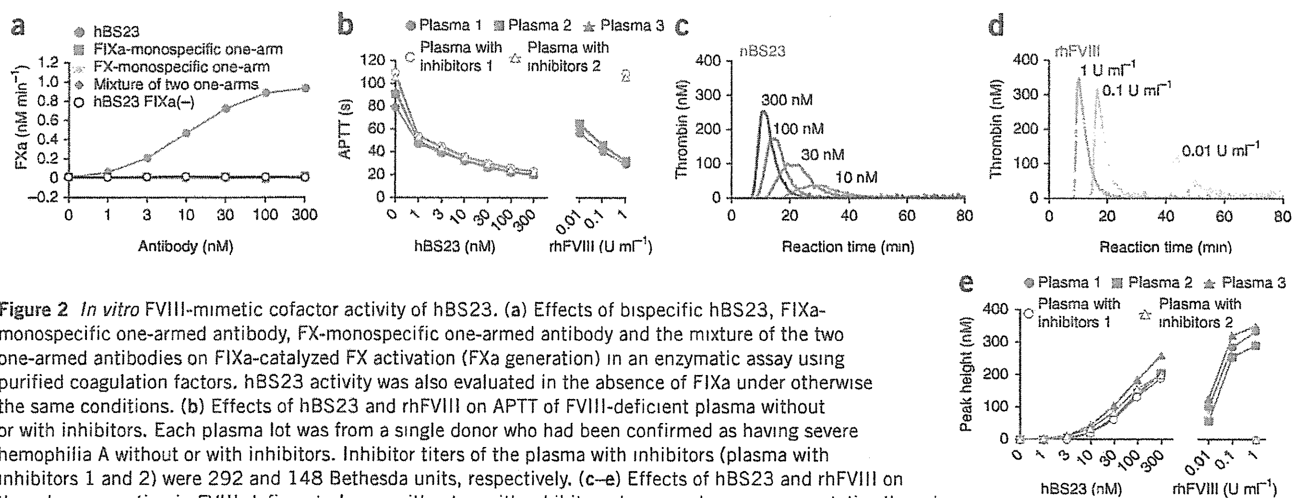

Figure 2 *In vitro* FVIII-mimetic cofactor activity of hBS23. (**a**) Effects of bispecific hBS23, FIXa-monospecific one-armed antibody, FX-monospecific one-armed antibody and the mixture of the two one-armed antibodies on FIXa-catalyzed FX activation (FXa generation) in an enzymatic assay using purified 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).

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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.

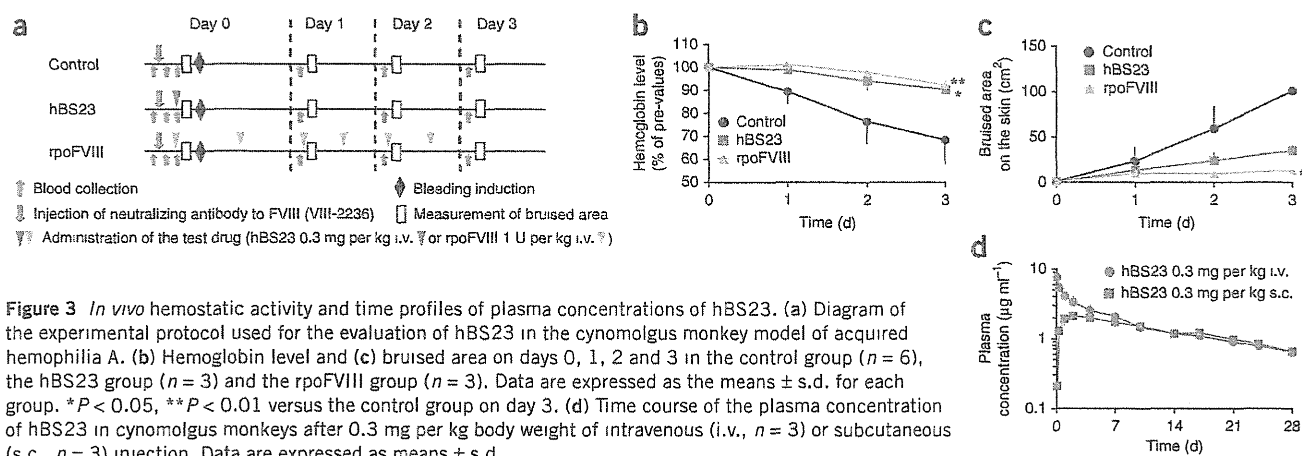

Figure 3 *In vivo* hemostatic activity and time profiles of plasma concentrations of hBS23. (**a**) Diagram of the experimental protocol used for the evaluation of hBS23 in the cynomolgus monkey model of acquired hemophilia A. (**b**) Hemoglobin level and (**c**) bruised area on days 0, 1, 2 and 3 in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

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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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1. Fischer, K., Lewandowski, D., Marijke van den Berg, H. & Janssen, M.P. Validity of assessing inhibitor development in haemophilia PUPs using registry data: the EUHASS project. *Haemophilia* **18**, e241–e246 (2012).
2. Oldenburg, J., El-Maarri, O. & Schwaab, R. Inhibitor development in correlation to factor VIII genotypes. *Haemophilia* **8** (suppl. 2), 23–29 (2002).
3. Berntorp, E. & Shapiro, A.D. Modern haemophilia care. *Lancet* **379**, 1447–1456 (2012).
4. Shi, Q., Kuether, E.L., Schroeder, J.A., Fahs, S.A. & Montgomery, R.R. Intravascular recovery of VWF and FVIII following intraperitoneal injection and differences from intravenous and subcutaneous injection in mice. *Haemophilia* **18**, 639–646 (2012).
5. Björkman, S. *et al.* Population pharmacokinetics of recombinant factor VIII: the relationships of pharmacokinetics to age and body weight. *Blood* **119**, 612–618 (2012).
6. Stonebraker, J.S., Bolton-Maggs, P.H., Soucie, J.M., Walker, I. & Brooker, M. A study of variations in the reported haemophilia A prevalence around the world. *Haemophilia* **16**, 20–32 (2010).
7. Geraghty, S. *et al.* Practice patterns in haemophilia A therapy—global progress towards optimal care. *Haemophilia* **12**, 75–81 (2006).
8. Manco-Johnson, M.J. *et al.* Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N. Engl. J. Med.* **357**, 535–544 (2007).
9. Young, G. *et al.* When should prophylaxis therapy in inhibitor patients be considered? *Haemophilia* **17**, e849–e857 (2011).
10. Astermark, J. *et al.* A randomized comparison of bypassing agents in hemophilia complicated by an inhibitor: the FEIBA NovoSeven Comparative (FENOC) Study. *Blood* **109**, 546–551 (2007).
11. Leissinger, C. *et al.* Anti-inhibitor coagulant complex prophylaxis in hemophilia with inhibitors. *N. Engl. J. Med.* **365**, 1684–1692 (2011).
12. Hay, C.R. & DiMichele, D.M. The principal results of the International Immune Tolerance Study: a randomized dose comparison. *Blood* **119**, 1335–1344 (2012).
13. Ragni, M.V. *et al.* Survey of current prophylaxis practices and bleeding characteristics of children with severe haemophilia A in US haemophilia treatment centres. *Haemophilia* **18**, 63–68 (2012).
14. Fay, P.J. Activation of factor VIII and mechanisms of cofactor action. *Blood Rev.* **18**, 1–15 (2004).
15. Lenting, P.J., Donath, M.J., van Mourik, J.A. & Mertens, K. Identification of a binding site for blood coagulation factor IXa on the light chain of human factor VIII. *J. Biol. Chem.* **269**, 7150–7155 (1994).
16. Fay, P.J. & Koshibu, K. The A2 subunit of factor VIIIa modulates the active site of factor IXa. *J. Biol. Chem.* **273**, 19049–19054 (1998).
17. Lapan, K.A. & Fay, P.J. Localization of a factor X interactive site in the A1 subunit of factor VIIIa. *J. Biol. Chem.* **272**, 2082–2088 (1997).
18. Liu, Z. *et al.* A potent erythropoietin-mimicking human antibody interacts through a novel binding site. *Blood* **110**, 2408–2413 (2007).
19. Mayorov, A.V. *et al.* Catalytic antibody degradation of ghrelin increases whole-body metabolic rate and reduces refeeding in fasting mice. *Proc. Natl. Acad. Sci. USA* **105**, 17487–17492 (2008).
20. Bhaskar, V. *et al.* A fully human, allosteric monoclonal antibody that activates the insulin receptor and improves glycemic control. *Diabetes* **61**, 1263–1271 (2012).
21. Beck, A., Wurch, T., Bailly, C. & Corvaia, N. Strategies and challenges for the next generation of therapeutic antibodies. *Nat. Rev. Immunol.* **10**, 345–352 (2010).
22. Baeuerle, P.A., Kufer, P. & Bargou, R. BiTE: teaching antibodies to engage T-cells for cancer therapy. *Curr. Opin. Mol. Ther.* **11**, 22–30 (2009).
23. Jackman, J. *et al.* Development of a two-part strategy to identify a therapeutic human bispecific antibody that inhibits IgE receptor signaling. *J. Biol. Chem.* **285**, 20850–20859 (2010).
24. Shen, B.W. *et al.* The tertiary structure and domain organization of coagulation factor VIII. *Blood* **111**, 1240–1247 (2008).
25. Saphire, E.O. *et al.* Contrasting IgG structures reveal extreme asymmetry and flexibility. *J. Mol. Biol.* **319**, 9–18 (2002).
26. Baker, M.P., Reynolds, H.M., Lumicisi, B. & Bryson, C.J. Immunogenicity of protein therapeutics: the key causes, consequences and challenges. *Self Nonself* **1**, 314–322 (2010).
27. Wang, W., Wang, E.Q. & Balthasar, J.P. Monoclonal antibody pharmacokinetics and pharmacodynamics. *Clin. Pharmacol. Ther.* **84**, 548–558 (2008).
28. Martens, T. *et al.* A novel one-armed anti-c-Met antibody inhibits glioblastoma growth in vivo. *Clin. Cancer Res.* **12**, 6144–6152 (2006).
29. Shima, M., Matsumoto, T. & Ogiwara, K. New assays for monitoring haemophilia treatment. *Haemophilia* **14** (suppl. 3), 83–92 (2008).
30. Shetty, S., Bhave, M. & Ghosh, K. Acquired hemophilia A: diagnosis, aetiology, clinical spectrum and treatment options. *Autoimmun. Rev.* **10**, 311–316 (2011).
31. Lin, Y.S. *et al.* Preclinical pharmacokinetics, interspecies scaling, and tissue distribution of a humanized monoclonal antibody against vascular endothelial growth factor. *J. Pharmacol. Exp. Ther.* **288**, 371–378 (1999).
32. Benincosa, L.J. *et al.* Pharmacokinetics and pharmacodynamics of a humanized monoclonal antibody to factor IX in cynomolgus monkeys. *J. Pharmacol. Exp. Ther.* **292**, 810–816 (2000).
33. Deng, R. *et al.* Projecting human pharmacokinetics of therapeutic antibodies from nonclinical data: what have we learned? *MAbs* **3**, 61–66 (2011).
34. Lillicrap, D. Improvements in factor concentrates. *Curr. Opin. Hematol.* **17**, 393–397 (2010).
35. Powell, J.S. *et al.* Safety and prolonged activity of recombinant factor VIII Fc fusion protein in hemophilia A patients. *Blood* **119**, 3031–3037 (2012).
36. High, K.A. Gene therapy for haemophilia: a long and winding road. *J. Thromb. Haemost.* **9** (suppl. 1), 2–11 (2011).
37. Harding, F.A., Stickler, M.M., Razo, J. & DuBridge, R.B. The immunogenicity of humanized and fully human antibodies: residual immunogenicity resides in the CDR regions. *MAbs* **2**, 256–265 (2010).
38. Merchant, A.M. *et al.* An efficient route to human bispecific IgG. *Nat. Biotechnol.* **16**, 677–681 (1998).
39. Igawa, T. *et al.* Engineering the variable region of therapeutic IgG antibodies. *MAbs* **3**, 243–252 (2011).
40. Igawa, T. *et al.* Reduced elimination of IgG antibodies by engineering the variable region. *Protein Eng. Des. Sel.* **23**, 385–392 (2010).

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

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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).

41. Bloom, J.W., Madanat, M.S., Marriott, D., Wong, T. & Chan, S.Y. Intrachain disulfide bond in the core hinge region of human IgG4. *Protein Sci.* **6**, 407–415 (1997).
42. Okuda, M. & Yamamoto, Y. Usefulness of synthetic phospholipid in measurement of activated partial thromboplastin time: a new preparation procedure to reduce batch difference. *Clin. Lab. Haematol.* **26**, 215–223 (2004).
43. Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S. & Winter, G. Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* **321**, 522–525 (1986).
44. Healey, J.F., Lubin, I.M. & Lollar, P. The cDNA and derived amino acid sequence of porcine factor VIII. *Blood* **88**, 4209–4214 (1996).
45. Yonemura, H. *et al.* Efficient production of recombinant human factor VIII by co-expression of the heavy and light chains. *Protein Eng.* **6**, 669–674 (1993).
46. Hemker, H.C. *et al.* Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol. Haemost. Thromb.* **33**, 4–15 (2003).

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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.

References

1 Davis J, Cheng AC, McMillan M, Humphrey AB, Stephens DP, Anstey NM. Sepsis in the tropical Top End of Australia's Northern Territory: disease burden and impact on indigenous Australians. *Med J Aust* 2011; 194: 519–24.
2 Cheng AC, Currie B. Melioidosis: epidemiology, pathophysiology and management. *Clin Microbiol Rev* 2005; 18: 383–416.
3 Currie BJ, Ward L, Cheng AC. The epidemiology and clinical spectrum of melioidosis: 540 cases from the 20 year Darwin prospective study. *PLoS Negl Trop Dis* 2010; 4: e900.
4 Morse LP, Moller C-C, Harvey E *et al.* Prostatic abscess due to *Burkholderia pseudomallei*: 81 cases from a 19-year prospective melioidosis study. *J Urol* 2009; 182: 542–7.
5 Huth-Kühne A, Baudo F, Collins P *et al.* International recommendations on the diagnosis and treatment of patients with acquired hemophilia A. *Haematologica* 2009; 94: 566–75.
6 Campos-de-Magalhaes M, Eduardo Brandao-Mello C *et al.* Factor VIII and IX deficiencies related to acquired inhibitors in a patient with chronic hepatitis C virus infection receiving treatment with pegylated interferon plus ribavirin. *Hematology* 2011; 16: 80–5.
7 Imbach P, Lazarus AH, Kuhne T. Intravenous immunoglobulin induce potentially synergistic immunomodulations in autoimmune disorders. *Vox Sang* 2010; 98: 385–94.

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

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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.

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

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 $4{,}830 \times 10^9$/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

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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.

References

1 DiMichele D. Immune tolerance induction in haemophilia: evidence and the way forward. *J Thromb Haemost* 2011; 9(Suppl 1): 216–25.

2 Ohga S, Nomura A, Takada H, Suga N, Hara T. Successful self-infusion of activated prothrombin complex concentrate for prophylaxis in a child with a factor VIII inhibitor. *Am J Hematol* 2007; 82: 145–9.

3 Shima M, Sawamoto Y, Nakai H *et al.* Measurement of anti-factor VIII IgG, IgG4 and IgM alloantibodies in previously untreated haemophilia A patients treated with recombinant factor VIII. Kogenate Japanese Clinical Study Group. *Int J Hematol* 1995; 62: 35–43.

4 Watanabe T, Watanabe H, Onishi T, Okamura K, Kgami S, Yamada T *et al.* Reduction of anti-FIX inhibitor titre in a haemophilia B patient with continuous use of activated prothrombin complex concentrate. *J Thromb Haemost* 2007; 5(Suppl 2): P-W-154 Abstract (XXIst ISTH Congress, August 2007)

5 Yoshioka A, Kamisue S, Tanaka I, Kato M, Kohmura I, Shima M *et al.* Anamnestic response following infusion of prothrombin complex concentrates (PCC) and activated prothrombin complex concentrates (APCC) in haemophilia A patients with inhibitors. *Blood Coagul Fibrinolysis* 1991; 2(Suppl 1): 51–58.

6 Jiménez-C V, Alvarez MT, Martin-Salces M *et al.* Prophylaxis in 10 patients with severe haemophilia A and inhibitor: different approaches for different clinical situations. *Haemophilia* 2009; 15: 203–9.

7 Holme PA, Glomstein A, Grønhaug S, Tjønnfjord GE. Home treatment with bypassing products in inhibitor patients: a 7.5-year experience. *Haemophilia* 2009; 15: 727–32.

8 Valentino LA. Assessing the benefits of FEIBA prophylaxis in haemophilia patients with inhibitors. *Haemophilia* 2010; 16: 263–71.

9 Lin PC, Liao YM, Tsai SP, Chang TT. Immune tolerance induction therapy for patients with haemophilia A and FVIII inhibitors particularly using low-dose regimens. *Pediatr Blood Cancer* 2011; 57: 1029–33.

10 Kurnik K, Bidlingmaier C, Engl W, Chehadeh H, Reipert B, Auerswald G. New early prophylaxis regimen that avoids immunological danger signals can reduce FVIII inhibitor development. *Haemophilia* 2010; 16: 256–62.

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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/

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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

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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

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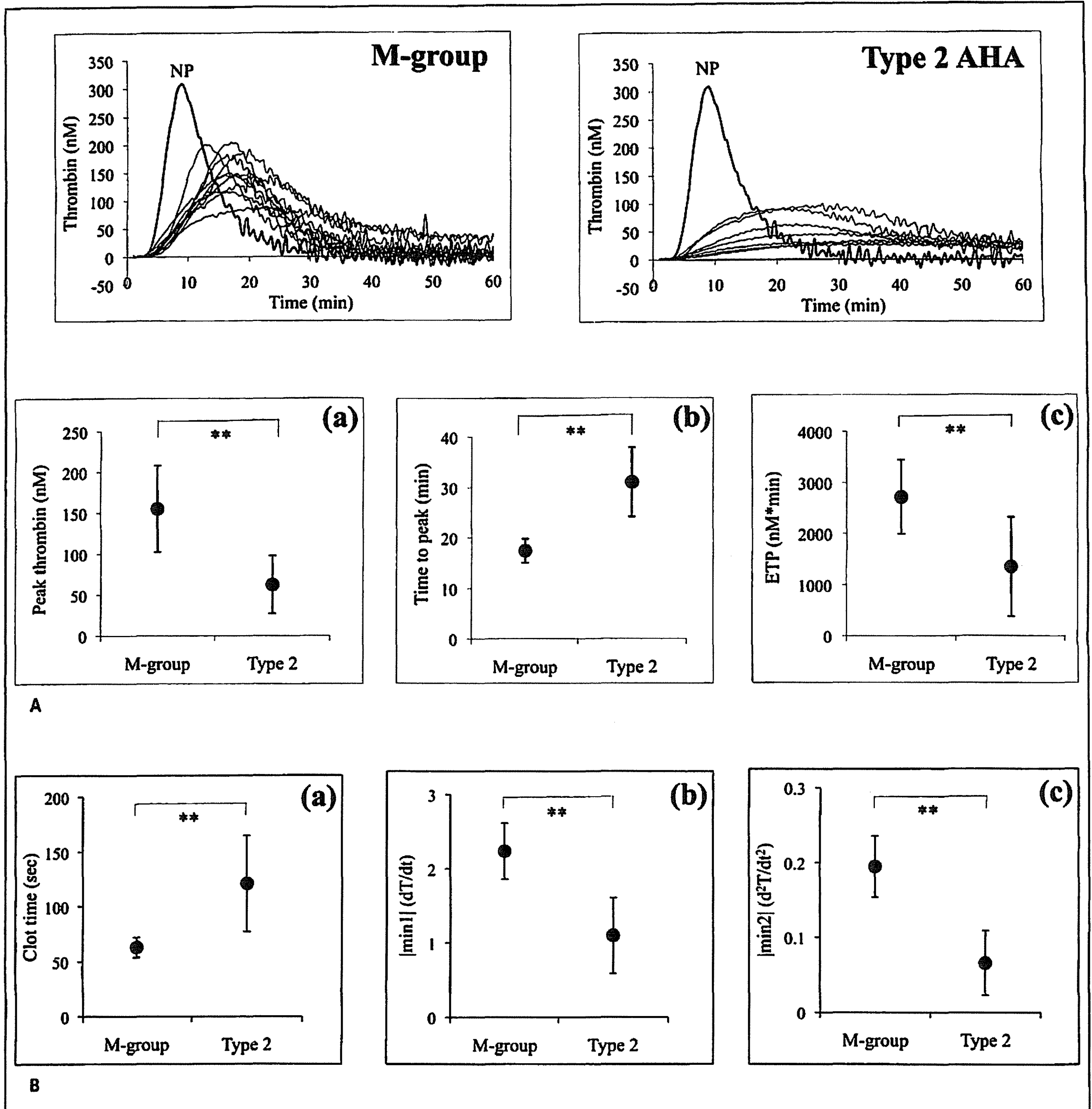

Figure 1: TGT and clot waveform analysis on patient's plasmas in the M-group and type 2 AHA. A) TGT-assay; upper panels: Plasma samples obtained from the M-group patients and type 2 AHA were preincubated with TF (0.5 pM), PL (4 µM) and ellagic acid (0.3 µM), followed by the addition of $CaCl_2$. Thrombin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

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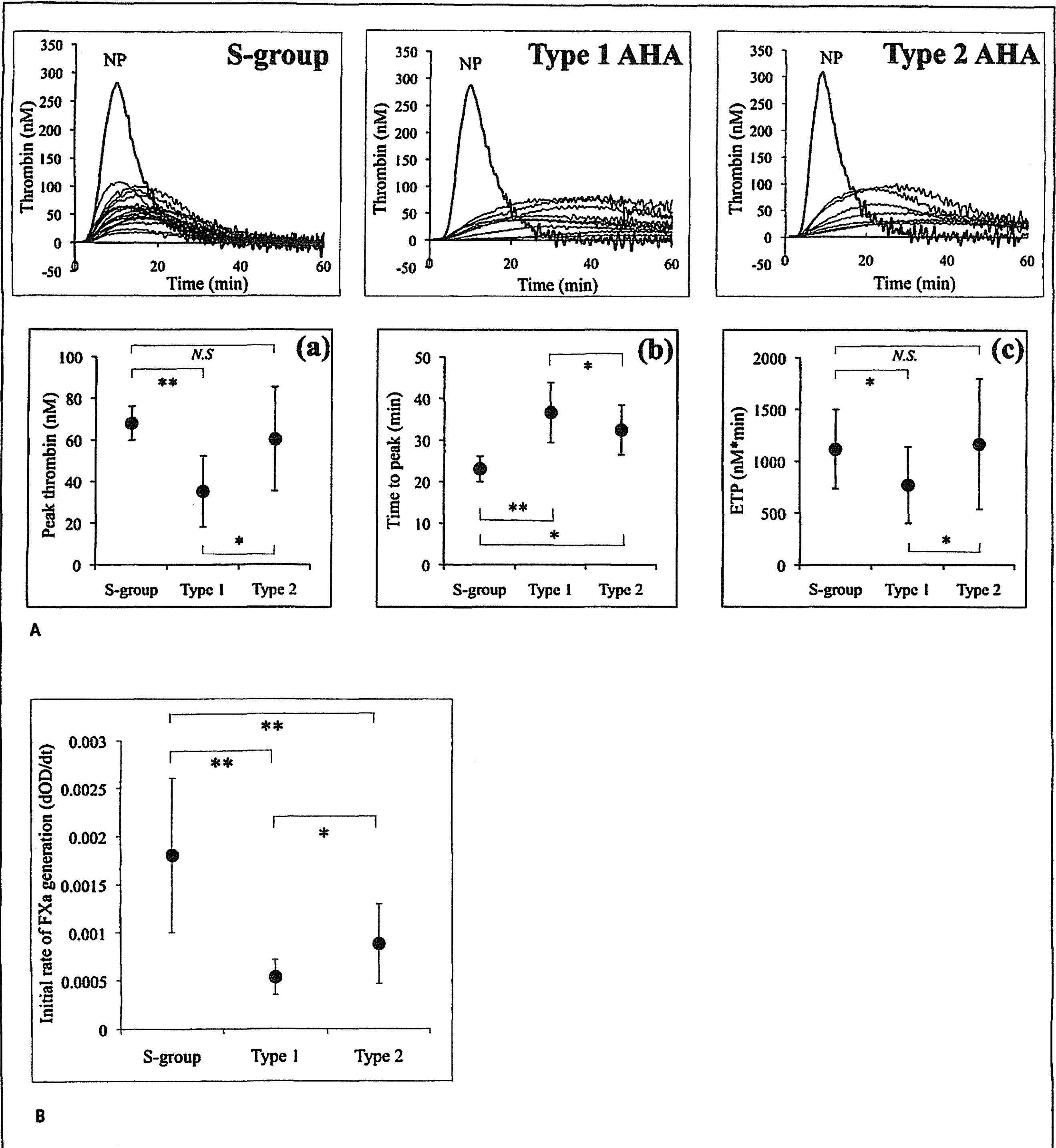

Figure 2: TGT and endogenous intrinsic FXa generation on patient's plasmas in the S-group, type 1, and type 2 AHA. A) TGT-assay; Upper panels: Patients' plasmas obtained from the S-group, type 1 AHA, and type 2 AHA were preincubated with TF (0.5 pM), PL (4 μM) and ellagic acid (0.3 μM), followed by the addition of $CaCl_2$. Thrombin generation was measured as described in *Methods*, and representative TGT curves are illustrated. NP; control normal plasma. Lower panels: The parameters of peak thrombin (a), time to peak (b), and ETP (c) were obtained from the TGT data shown in upper panels. B) Endogenous intrinsic FXa generation; Patients' plasmas obtained from S-group, type 1, and type 2 AHA were preincubated with FIXa/FX/PL mixture in the presence of hirudin, followed by the addition of $CaCl_2$ as described in *Methods*. FXa was measured using commercial reagents. The initial velocity rates of endogenous FXa generation are illustrated. In all instances, results are shown as mean ± SD from at least five separate experiments. The value of FVIII:C 1.0 IU/dl as a reference value was 5.04 ± 0.20 $\times 10^{-3}$. *p<0.05, **p<0.01, NS; no significance.

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~2.0-fold (121 ± 44/61 ± 8 seconds, $p<0.05$; *panel* a), compared to those in the M-group, and both |min1| and |min2| values were significantly decreased by ~2.1-fold, (1.1 ± 0.5/2.3 ± 0.3, $p<0.01$; *panel* b) and by ~3.1-fold (0.06 ± 0.03/0.19 ± 0.04, $p<0.01$; *panel* c), respectively. These results demonstrated that blood coagulation in type 2 was markedly more defective than in the M-group, despite similar FVIII:C levels (2.1 ± 0.9/2.0 ± 1.9 IU/dl, respectively). The findings were in keeping with the more severe haemorrhagic symptoms observed in type 2 relative to the M-group of patients.

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

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

Intrinsic FXa generation, corresponding to the upstream process of thrombin generation, was further examined to clarify the mechanism(s) of excessively defective thrombin generation in AHA. Plasma samples from each of the three groups were incubated with FIXa/FX/PL mixtures in the presence of hirudin (to eliminate thrombin reactions). $CaCl_2$ was added and endogenous intrinsic FXa generation was measured using the chromogenic assay. The initial rate of FXa generation was decreased in the order type 1, type 2, S-group (0.53 ± 0.18/0.88 ± 0.41/1.81 ± 0.78 $\times 10^{-3}$) with significant differences (▶Fig. 2B). These results were consistent with those obtained in the TGT, and further suggested that the discrepancies in coagulation function between AHA and S-group HA could be attributed to a significant decrease in the expression of intrinsic tenase complex activity (FVIIIa/FIXa/FX/PL).

Properties of anti-FVIII autoAbs in AHA

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

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

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

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

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

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

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

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

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

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

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

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

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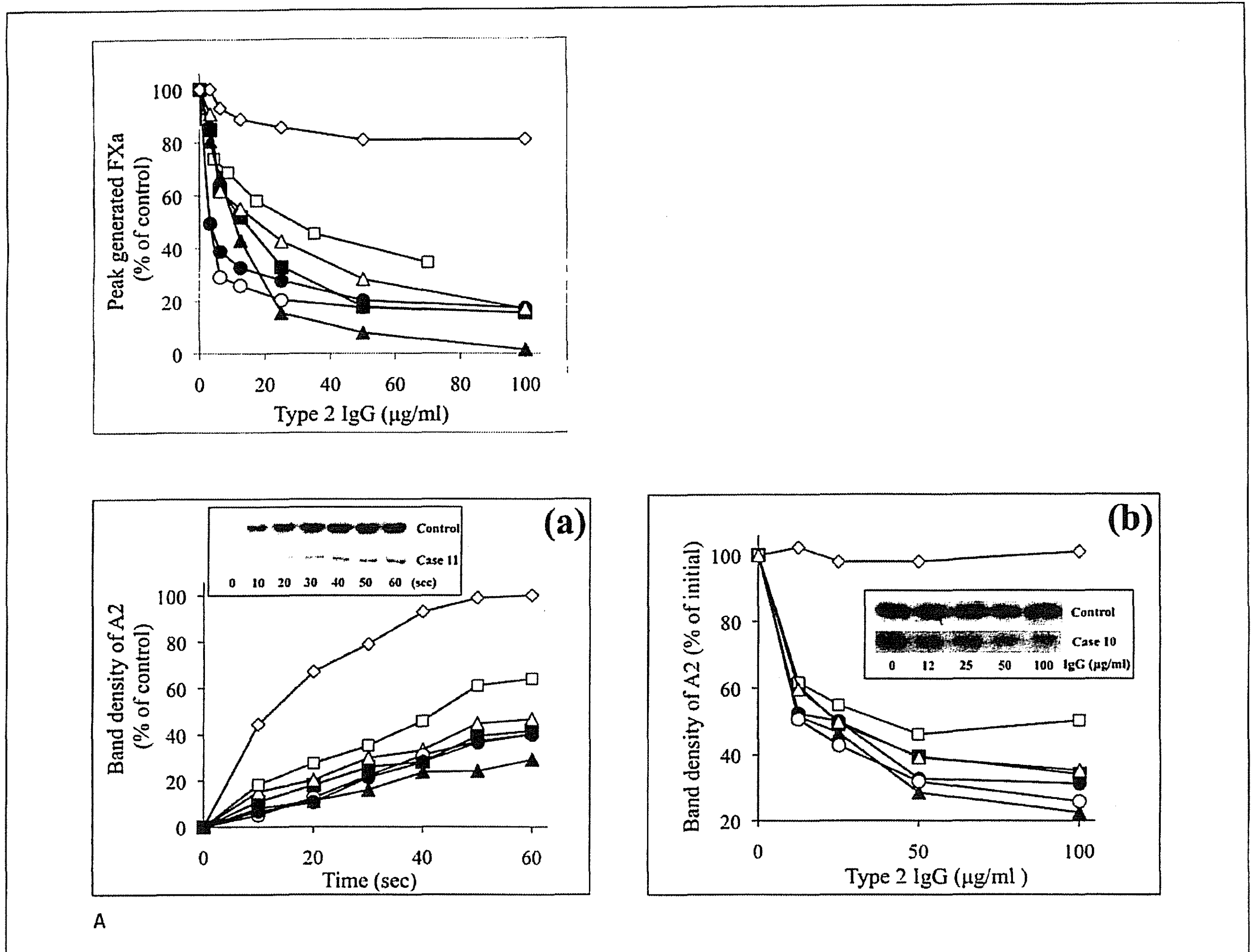

Figure 3: Effects of type 2 anti-C2 autoAbs on thrombin- or FXa-catalysed activation of FVIII. A) Thrombin reaction; Upper panel: FVIII (0.05 nM) was activated by thrombin (1 nM) for 1 min. After the addition of hirudin and dilution, FXa generation was initiated by the addition of FIXa (1 nM), FX (150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

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